PATHOLOGICAL TECHNIQUE

A Practical Manual for Workers in Pathological Histology and Bacteriology

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Directions for the Performance of Autopsies and for Clinical Diagnosis by Laboratory Methods

BY

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Eighth Edition
Revised and Enlarged
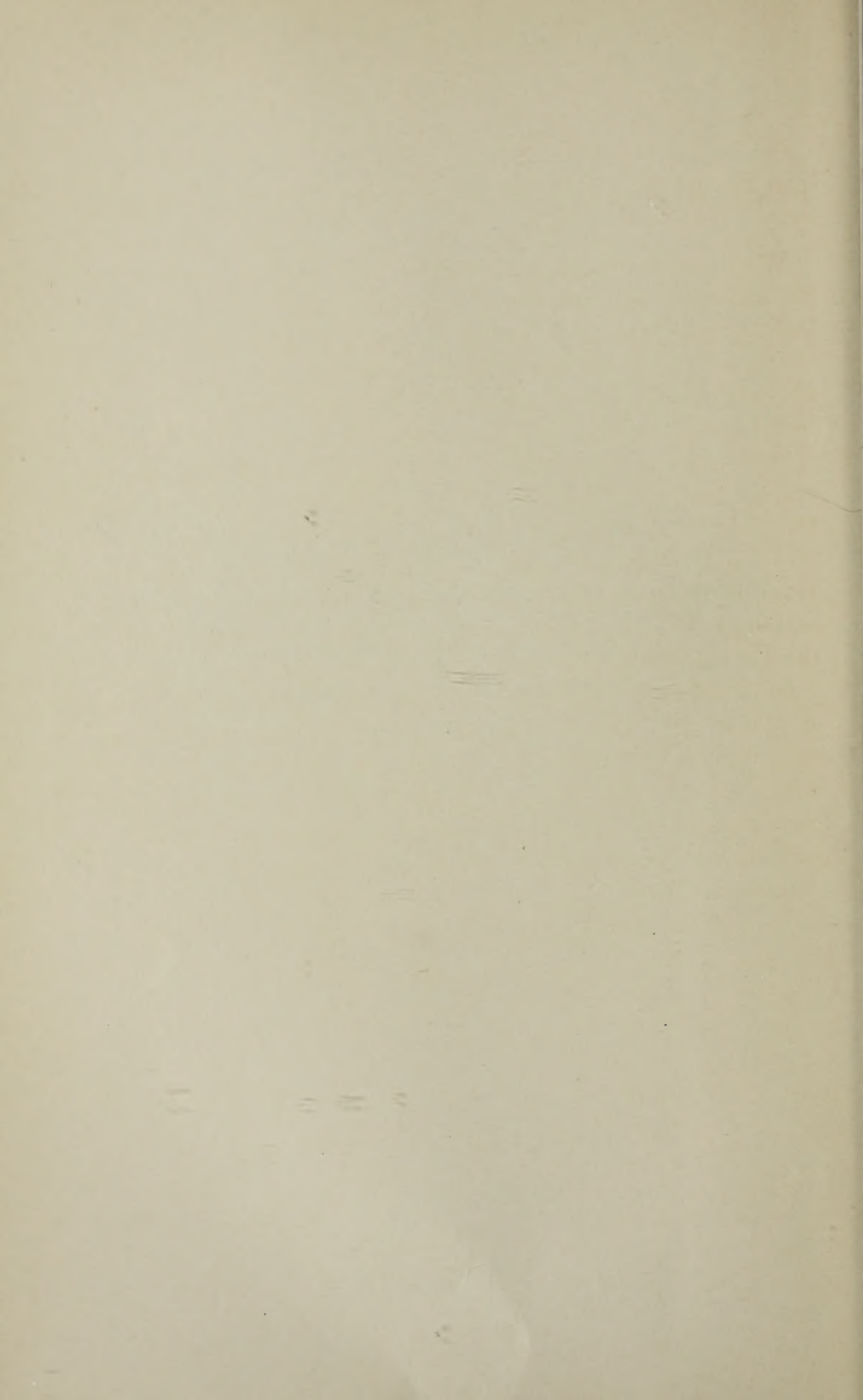With 180 Illustrations

TO

HENRY F. SEARS, A. M., M. D.,

WHO BY HIS LIBERALITY FIRST RENDERED POSSIBLE PATHOLOG-
ICAL RESEARCH IN BOSTON, AND BY HIS PERSONAL
WORK ADVANCED AND STIMULATED IT,

THIS BOOK IS RESPECTFULLY DEDICATED BY

THE AUTHORS

PREFACE TO THE EIGHTH EDITION

WE are greatly indebted to several generous collaborators for assistance in preparing the present edition of the Pathological Technique. Dr. Robert N. Nye has thoroughly revised the four divisions devoted to the subject of Bacteriology and also the section on Serum Diagnosis. The chapter devoted to the Blood has been rewritten and elaborated by Dr. Thomas E. Buckman with a view of presenting both the technique of examination and the clinical interpretation of the results. Dr. Samuel T. Orton has brought up to date the section on the Central Nervous System. Dr. James B. Ayer and Miss Jessie R. Cockrill have contributed the chapter on the Examination of the Spinal Fluid, and Dr. Donald Munro the one on the Method of Opening the Skull in the New-born.

Other sections which have been entirely rewritten are those on Pigments, Fats and Lipoids, and Museum Preparations. Brief directions in regard to Photography of Gross Pathological Specimens and on the Essentials of Photomicrography have been inserted, while a few paragraphs which seemed unnecessary, at least in their present state, have been omitted.

BOSTON, MASS.,
April, 1924.
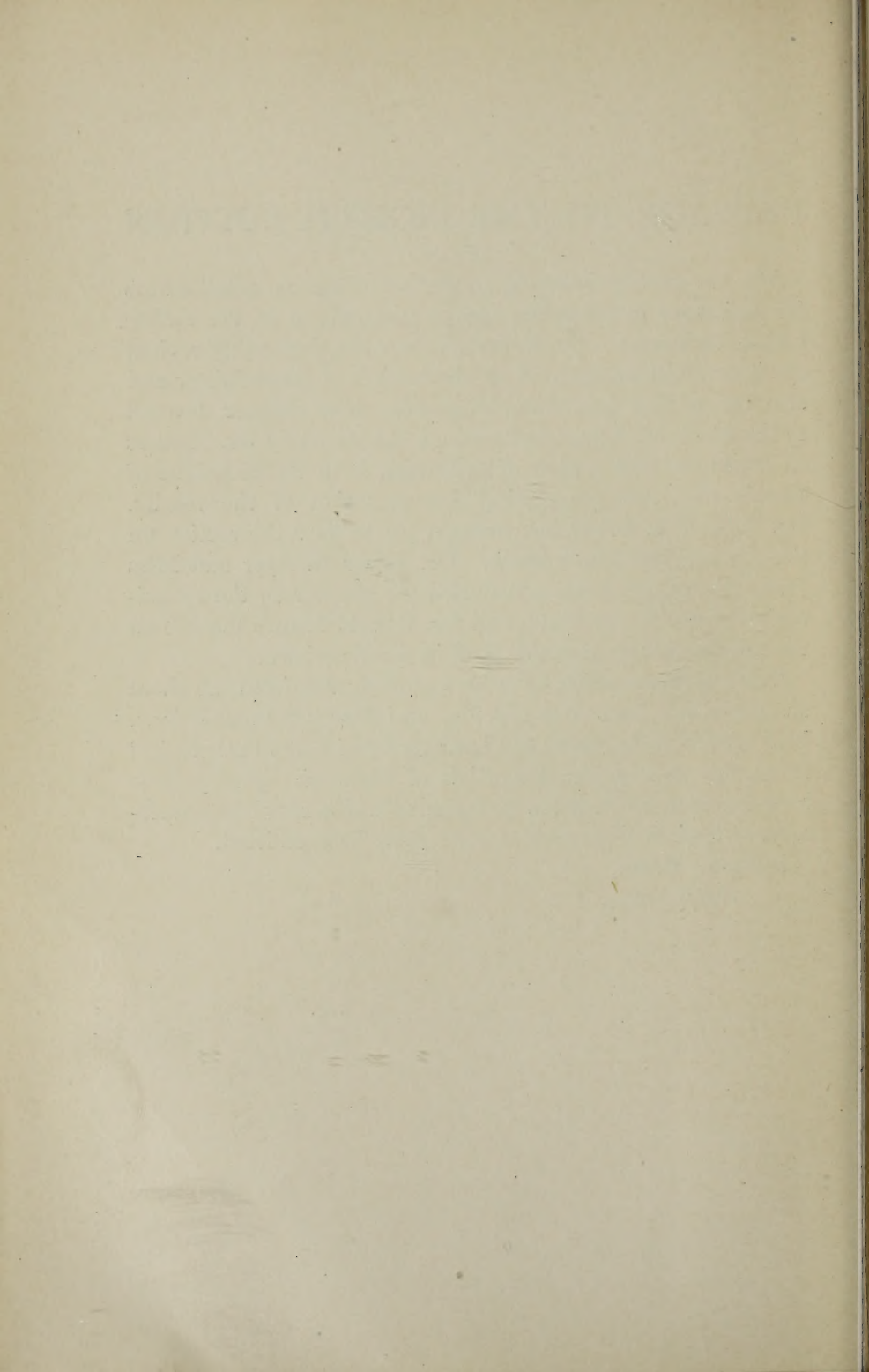
PREFACE TO THE FIRST EDITION

This book is designed especially for practical use in pathological laboratories, both as a guide to beginners and as a source of reference for the advanced. We believe that the book will also meet the wants of practitioners who have more or less opportunity to do general pathological work.

Every autopsy presents for solution a problem which may be simple or complex. The known quantities are certain clinical symptoms and physical signs; the unknown quantities are not only the gross and microscopic lesions that may or may not have given rise to the symptoms and signs, but also the etiology of the lesions and the order of their sequence. The solution of the problem often requires the highest skill in post-mortem, bacteriological, and histological technique, but in its solution lies the fascination of pathological work.

It has seemed advisable to us to present, so far as possible, a consecutive statement of the methods employed in solving the various problems that arise, so as to avoid the repetitions that necessarily occur when the three usual divisions of the subject are separately considered by different writers. It is hoped that this method of presenting the subject will bring the student to the realization that the mechanical performance of a post-mortem examination and the inspection of the gross lesions constitute usually only the beginning of the solution of the problem, which should be investigated bacteriologically, histologically, and chemically as far as our present knowledge will permit.

We should particularly advise the routine bacteriological and histological examination of the more important organs in all suitable cases. Naturally, the autopsies in which the lesions are due to a single etiological factor are the most valuable and instructive for a clear understanding of the pathological processes present.

Besides the methods of post-mortem examinations and of bacteriological and histological investigations connected with autopsies, we have added the special methods employed in clinical bacteriology and pathology.

In the parts devoted to Bacteriology and to Pathological Histology we have not endeavored to make an exhaustive collection of methods and formulæ, but rather to select those which have been found of the greatest service in practical work.

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Introduction.—The ideal function of the technique of pathological histology is so to fix tissues for microscopic examination that every tissue-element or pathological product is perfectly preserved with all its morphological and chemical properties intact, and so to stain tissues that every tissue-element or pathological product can be readily differentiated from any other tissue-element or pathological product that resembles it. In certain respects only has this ideal yet been reached, but the number of differential stains is increasing yearly.

In the following pages the various steps in the preparation and staining of tissues have been arranged, so far as possible, in logical sequence.

LABORATORY OUTFIT

The modern pathological laboratory, especially if connected with a hospital, requires in its outfit a considerable number of instruments and utensils owing to the variety of work which must be performed in it. It is not our function to appraise the relative merits of the different microscopes and microtomes, for example, which are on the market, but we shall mention certain ones which we have found from long-continued use to be most excellent. American microscopes are steadily improving, but have not yet reached the standard set by Zeiss, although more nearly approaching it every year. In other lines, such as microtomes, incubators,

and centrifuges, it is doubtful if our instruments can be excelled elsewhere.

Microscopes.—The most important laboratory instrument is the microscope. It should be, so far as means will permit, the best that skill can produce. Excellent microscopes are manufactured both abroad and in this country, but no make of microscope can be unconditionally recommended. Undoubtedly the best microscopes in every particular and the most expensive are those manufactured by Zeiss.

Microscopes made in the United States are better than they used to be and are steadily improving, but they are not yet perfect. Those manufactured by the Bausch & Lomb Optical Co. can be highly recommended.

It is important for a beginner in microscopy, before buying a microscope of any make, to have it carefully examined and its lenses tested at a pathological or other laboratory by some one skilled in its use. The continental form of stand of medium size is to be preferred to all others. The large stand is undesirable, because it is too heavy and too high for comfortable use. It should be furnished with rack and pinion, and with micrometer screw for coarse and fine adjustment, with a triple or quadruple nose-piece, and with an Abbé condenser and iris diaphragm. The necessary objectives are a low and a high dry, and a $\frac{1}{12}$ oil-immersion. Two eye-pieces, a low and a high, will be found sufficient for all ordinary purposes.

The stands, oculars, and objectives of the Zeiss make generally used are the following:

Stands, III and IV.

Oculars, 2, 3 and 4.

Objectives, A, C, D, and $\frac{1}{12}$ oil-immersion.

Or in the apochromatic series,

Oculars, 4, 6, and 8.

Objectives, 16.0, 8.0, 4.0, and oil-immersion 2.0 mm., apert. 1.30.

Even if all these different parts cannot be purchased at the same time, it is important to buy a stand to which they

afterward may be added, for the list includes only what every medical practitioner should have at his service for the proper examination of urine, sputum, blood, etc.

The apochromatic lenses and compensation oculars are too expensive to come into general use. Fortunately, they are more important for photomicrography than for general microscopic work.

The *oil-immersion lens* should always be cleaned after using by wiping off the oil with the fine lens-paper now manufactured for that purpose. If the lens is sticky, moisten the paper with benzol or xylol. The same process can be used if necessary for the dry lenses, but it must be done quickly, so as not to soften the balsam in which the lenses are embedded. Ordinarily breathing on the lens and wiping with lens-paper is sufficient.

In using the *Abbé illuminating apparatus* it is important to bear in mind that the best results are obtained, according to Zeiss, by employing the plain mirror, for the condenser is designed for parallel rays of light. The concave mirror is to be used only when some near object, such as the window-frame, is reflected into the field of vision or when artificial light is employed.

A *mechanical stage* is now made which can be instantly attached to any microscope. It is exceedingly useful for blood-counting or for searching carefully the whole surface of a stained cover-slip. For ordinary work it is undesirable.

Illumination.—For microscopic work the best illumination is that obtained from a white cloud, although for some purposes the light which filters through a white curtain on which the sun is shining is very useful, especially with the highest powers of the microscope. When artificial light is necessary, the Welsbach burner, or, better still, a tungsten electric light with ground-glass globe, will be found very satisfactory. The slightly yellowish tint of the light can be corrected, if necessary, by means of a piece of blue glass or, better still, of the new daylight glass inserted beneath the Abbé condensor. Of the different electric lamps designed for use with the microscope that put out by Leitz is so far the simplest, best, and most

powerful. It is advisable to use the large form designed for dark-field illumination and furnished with the 100 Watts nitrogen filled tungsten bulb so as to be able to obtain plenty of light for oil-immersion work. Inasmuch as the large daylight glass filter which goes with it is very liable to break, it seems better to replace it with the ground glass and to insert a small circle of daylight glass below the Abbé condensor.

For drawing, the *Abbé camera lucida* will be found extremely useful and convenient.

Much use is also made of a vertical projection apparatus for the same purpose, especially when only outline drawings are required. For fine details it is not so good.

Microtomes.—Three different kinds of microtomes are required in laboratory work. They are known as the freezing microtome, the celloidin microtome, and the paraffin microtome. Each has its own special field of usefulness.

Freezing Microtome.—Freezing by means of the evaporation of ether, more rarely of rhigolene, was originally the method in general use. The process was both expensive and slow. A much cheaper and more rapid method of freezing was originated many years ago in the Pathological Laboratory of the Harvard Medical School by Dr. S. J. Mixter, and has since been universally adopted. This method consists in the employment of compressed carbon dioxid which is found in commerce in iron cylinders containing each about twenty pounds of liquefied gas. It is commonly used for charging beer and soda-water. As a rule, the cylinders are loaned, so that it is necessary to pay for the contents only.

The cylinder must be securely fastened in an upright position near the microtome, with its valve end below and with its escape-tube on a level with the entrance-tube into the freezing-box. The cap covering the escape-tube of the cylinder should have a small hole bored through it, and into this hole a small brass tube about 5 cm. long, with a fine bore, should be tightly driven. This permits the use of a smaller stream of gas than the escape-pipe of the cylinder would otherwise furnish. The same cap can be kept to use on all future cylinders.

The cylinder is connected with the microtome by means of a short piece of thick, strong rubber-tubing with small bore, so as to fit snugly over the escape-tube of the cylinder and the entrance-tube into the freezing-box. It is advisable to wire each end of the rubber-tubing around the tube it incloses. The connection can also be made with flexible copper tubing.
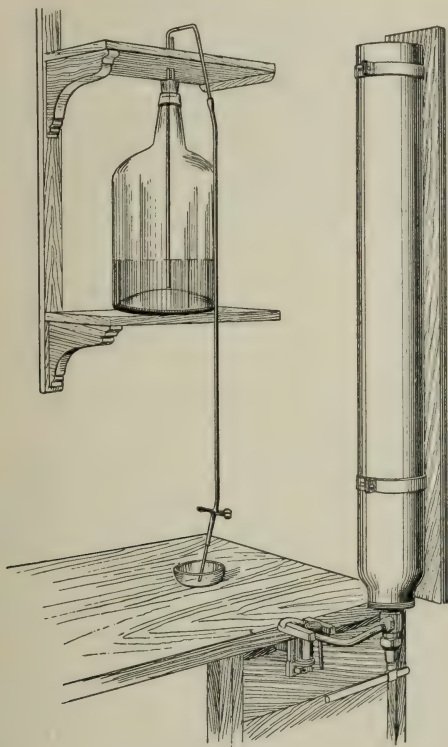
FIG. 1.—Freezing microtome.

In order to obtain better leverage and more perfect control over the escape of the gas than are needed for the purposes for which the cylinders are ordinarily used, it is necessary to lengthen to about 25 cm., in whatever way seems best, the handle of the key which opens the escape-valve.

The first time the cylinder is used for freezing, a little water may escape, causing considerable sputtering. In freezing, the

valve should be turned carefully, so that the gas may escape slowly and evenly. Tissues fixed by alcohol or any other reagent, except formaldehyd, must be washed in running water for some hours before they can be frozen.

Even for tissue fixed in formaldehyd washing in water for ten to thirty minutes is advisable, as better sections can be obtained.

It is now possible to obtain from stores carrying automobile supplies small tubes of compressed carbon dioxid sufficient for one or two freezings. They will be found convenient for carrying to private operations when an immediate diagnosis by means of frozen sections is demanded.

Several forms of the freezing microtome are on the market. Of the simple types, the Bausch & Lomb table microtome can be recommended.

For cutting frozen sections on this type of instrument the blade of a carpenter's plane, $2\frac{5}{8}$ inches wide, mounted in a wooden handle (Fig. 2), will be found very serviceable and easy to sharpen.

Fig. 2.—Knife for freezing microtome, made from the blade of a carpenter's plane.

Of the more complicated freezing microtomes that manufactured by the Spencer Lens Co. can be highly recommended.

Celloidin Microtome.—There are two types of celloidin microtomes—one in which the object is raised by a screw, a second in which the object is raised by being moved up an inclined plane. The first type of machine is the better, for two reasons: the screw affords greater accuracy in the even elevation of the object than is possible with an inclined plane, and the object remains at all times in the same relative position with regard to the knife, so that an equally long sweep of the blade can be obtained for every section. An excellent instrument of this type is made by Bausch & Lomb (Fig. 3). For practical work it is much to be preferred to the elaborate Schiefferdecker-Becker microtome, designed for cutting sections under alcohol.

A new and wholly original microtome, in which the knife remains fixed and is clamped at both ends, while the object-
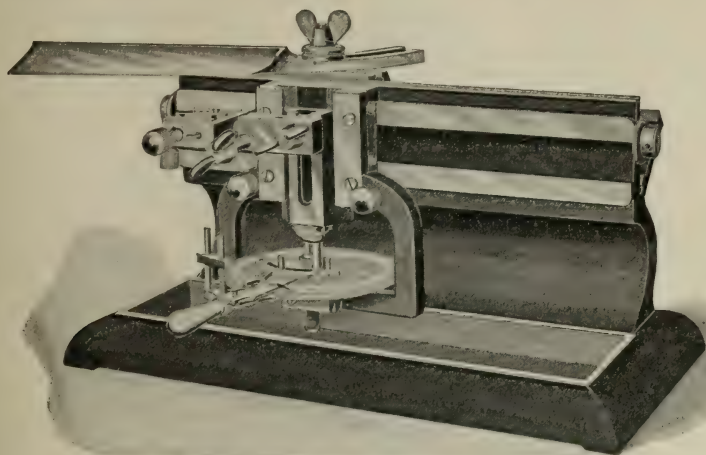
FIG. 3.—Large laboratory microtome (Bausch & Lomb).

holder, which is raised by a screw, moves back and forth beneath the knife, has recently been designed by Dr. C. S.
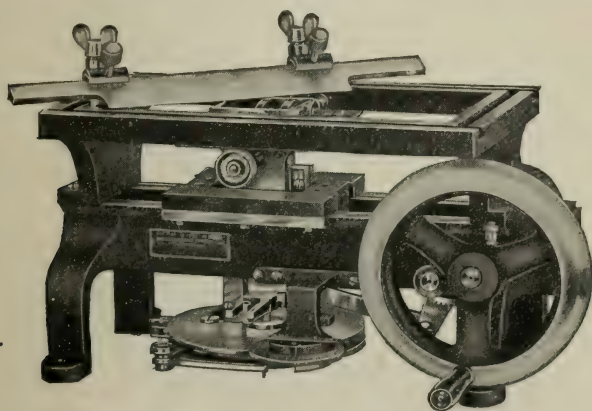
FIG. 4.—Minot precision microtome (Bausch & Lomb).

Minot and is manufactured by Bausch & Lomb (Fig. 4). It is intended both for celloidin and for paraffin work.

A *drop-bottle* on an elevated stand, with screw arrangement for regulating the amount of alcohol, is the most convenient

method for keeping the object and the knife wet while cutting; 80 per cent. alcohol should be used.
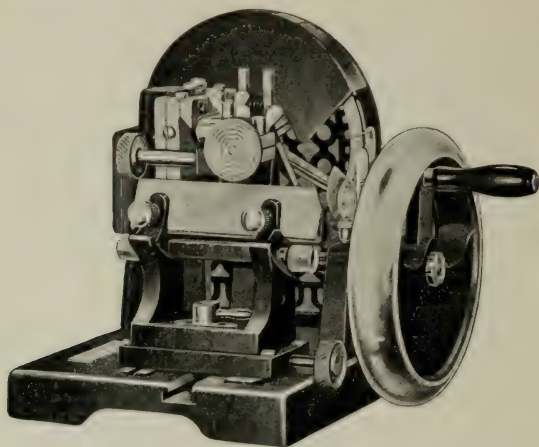
FIG. 5.—Minot's wheel microtome (Bausch & Lomb).

Paraffin Microtome.—Although paraffin sections can be cut on a celloidin microtome, it is preferable to have an instrument designed for the purpose. Two models of the Minot
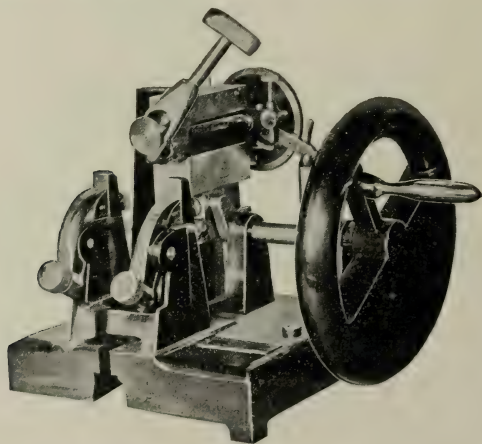
FIG. 6.—Minot rotary microtome (International Equipment Co., Boston, Mass.).

wheel microtome are manufactured in this country: one by the Bausch & Lomb Optical Co., the other by the International Equipment Co., of Boston, Mass. The latter instrument has

this advantage for pathological work: it is simple and heavy in construction, and the paraffin block-holder is controlled by a ball-and-socket joint, requiring but one screw instead of three. It has been found very satisfactory in practical use.

Paraffin Bath.—The best bath for keeping paraffin at a constant temperature is a thermostat of suitable size with hot-water jacket, such as is used for growing cultures of bacteria. The paraffin is kept in it on shelves in glass dishes of various sizes. The advantages of this method over the old way of using copper cups set into the top of a water-bath are that the paraffin is kept absolutely free from dust, each worker can have his own set of dishes, and the smallest bits of tissue can be readily found in them, because they are transparent.

A preliminary bath of soft paraffin is wholly unnecessary, and only prolongs the objectionable stage of heating. The regulator should register only one or two degrees above the melting-point of the paraffin.

Paraffin should be melted and decanted or filtered before use, as it often contains foreign material. When hot, it runs through an ordinary filter without trouble. A hot-water jacket to the funnel is not at all necessary.

Centrifuge.—This instrument is of great use in obtaining quickly the sediment from various fluids, including blood and urine, and also for sedimenting and washing the red blood-corpuscles used in the Wassermann and Noguchi serum tests. The electrically run instruments manufactured by the International Equipment Co., of Boston, Mass., can be highly recommended as well made, durable, and easy running. Size 1, type B, with 8-tube, 50 c.c. head will be found very satisfactory.

Vulcanized Fiber.—For mounting celloidin preparations nothing is so poor as cork, although it has been in use for years. The chief objections to it are that it does not furnish a rigid support to the embedded object; that, unless weighted, it floats in alcohol with the specimen downward; and that it yields a coloring material which stains both the alcohol and the specimen. Wood is not much better, although, of course, much firmer. Glass blocks have been proposed, and might do fairly

well if there did not exist an ideal substance—viz., vulcanized fiber. This can be obtained in boards or strips, preferably $\frac{1}{2}$ or $\frac{5}{8}$ inch in thickness, and sawn to any desired dimensions.

FIG. 7.—Centrifuge, size 1, type B, with 8-tube, 50 c.c. head manufactured by the International Equipment Co.

It is perfectly rigid, is heavy enough to sink specimens to the bottom of the jar in an upright position, is unaffected by alcohol or water, is light red in color, so that it is easily written on with
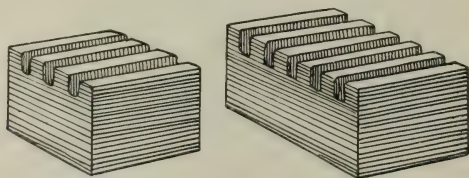
FIG. 8.—Blocks of vulcanized fiber.

a lead pencil, gives off no coloring material, and is practically indestructible.

Several parallel cuts, 1 to 2 mm. in depth, should be sawn into the upper surface of each block, so as to give the celloidin a firm hold.

Knives.—The knives for both the celloidin and the paraffin microtomes should be heavy and not too long, so as to afford as great rigidity as possible; they should be biconcave, so that they may be easily sharpened. It is important that every one
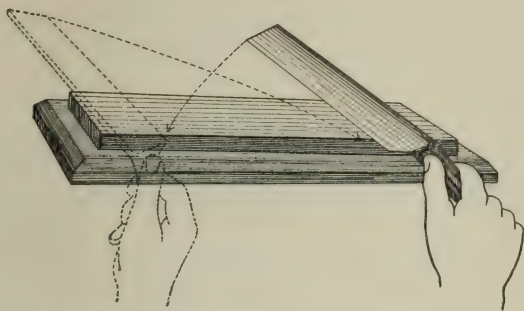
FIG. 9.—Diagram of the direction of the movements in honing.

who does much work in a pathological laboratory should learn to sharpen his own knives. The requisite skill is not difficult to acquire, and the time spent in learning is fully compensated
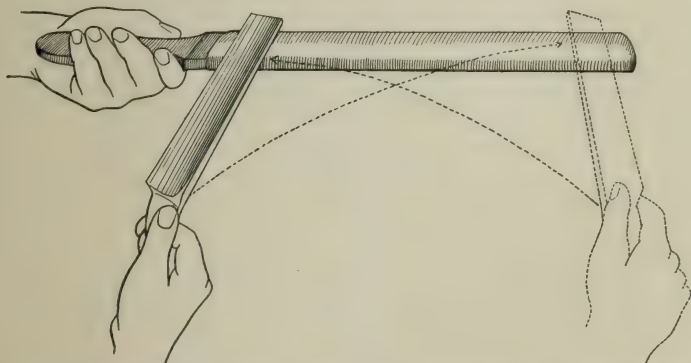
FIG. 10.—Diagram of the direction of the movements in stropping.

for by the ability always to have a sharp knife when it is wanted. For honing a knife either a fine yellow Belgian water-stone or a glass plate with diamantine and Vienna chalk may be used. A very fine carborundum "sixty seconds" stone that

has recently been made is advisable for removing nicks. In honing, the edge of the knife is forward and the motion is from heel to toe. The knife should always be turned on its back, and the pressure on it should be at all times rather light.

In stropping, the movement is reversed. The back of the knife necessarily precedes the edge. and the motion is from toe to heel. The direction of the movements in honing and stropping is best illustrated by the diagrams (Figs. 9, 10).

The condition of the cutting edge can be examined by drawing the knife flatwise across the low power field of the microscope. When the knife is properly sharpened the edge is smooth and even, without nicks.

A razor-strop paste greatly facilitates the smoothing of the knife edge in stropping.

Gillette Safety Razor blades, used with a suitable holder, give satisfactory paraffin sections. Success in the use of these blades depends on careful adjustment of the blade in the holder so that the edge of the holder bears exactly on the beginning of the bevel of the blade. A holder designed by J. H. Wright may be obtained from the International Equipment Co.

Running water for washing out specimens which have been fixed in Zenker's and other solutions is most easily supplied by having a water-pipe, furnished with numerous cocks 5–10 cm. apart, run horizontally over a slightly sloping shelf adjoining the sink. Attached to each cock is a rubber tube, with a glass tube in the end of it long enough to reach to the bottom of the jar (Fig. 11). By this arrangement the amount of water supplied to each specimen can be easily regulated.

Slides should be of colorless glass with ground edges. The English form, measuring 1 × 3 inches (76 × 26 mm.), is to be preferred for ordinary use. Occasionally broader slides are needed. Thick slides are preferable to thin ones; the latter are so light that they are easily lifted by the oil-immersion lens; they also seem to warp when heated to attach paraffin sections to them. They break readily if too much pressure is applied in wiping or rubbing them.

Cover=slips should be square or oblong according to the shape of the specimen. Most dry lenses are adjusted for cover-glasses measuring 16 or 17μ in thickness, so that if possible no cover-slip ranging outside of 15 to 18μ should be used. With an oil-immersion the exact thickness is not quite so important except that they be thin enough.

Slides and cover-slips are cleaned by dipping in alcohol and wiping dry with a soft crash towel or old linen handkerchief.
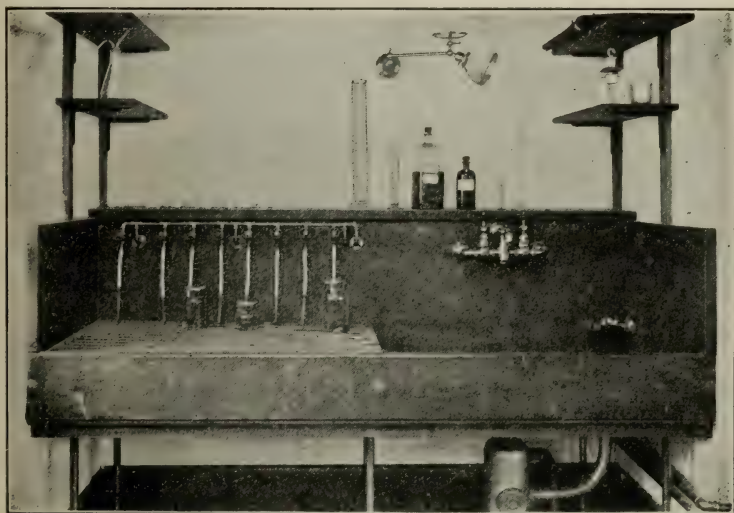
FIG. 11.—Large laboratory sink, showing adjoining shelf and arrangement for running water.

Cover-slips, after they are clean, should be preserved dry in covered dishes. The common method of keeping them under alcohol cannot be recommended.

Staining Dishes.—Watch-glasses are not satisfactory on account of their instability. Concave dishes with flat bottoms are much better for ordinary use, and can be obtained of several patterns. They should be large enough to hold 25 c.c. of fluid. The *Syracuse solid watch-glasses* are very good dishes of this shape. Individual glass butter dishes can be obtained which are very satisfactory and comparatively cheap.

Stender dishes of various sizes will be found useful for many purposes.

The "*New Practical Staining Dish*" manufactured by the Bausch and Lomb Optical Co. is very useful for staining at once a number of paraffin sections.
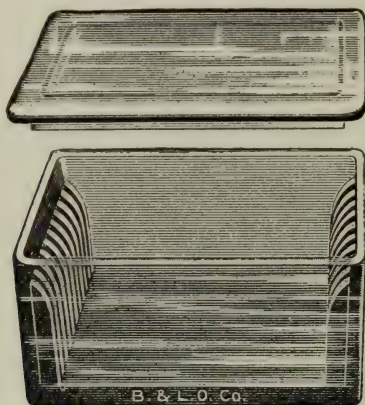
Fig. 12.—New practical staining dish.

Oblong rectangular Petri dishes are very convenient for staining preparations mounted on the slide.

For staining at once a large number of sections for class purposes, the *Hobb's Tea Infuser* has been found very useful. If set in a small lemonade cup, but little stain is required.

Large concave dishes holding 200 c.c. will be found the most convenient for holding frozen sections of fresh tissue, because a slide can be dipped into them and under the sections. They are known in the trade as glass nappies.

Large flat-bottomed glass dishes known as *crystallizing dishes*, holding one to three liters, are excellent to fix tissues in, as they allow the thin slices of material to lie flat. If several sizes are obtained the large dishes serve as good covers for the smaller ones.

Metal Instruments.—Spatulas of different sizes are needed. They should be thin, smooth, and large enough, so that a section will not curl over the edge (Fig. 13).

The best instrument for transferring sections under all circumstances is a piece of platinum wire mounted in an ordinary screw needle-holder. It is pliable and can be bent to any shape, will not break like a glass needle when dropped, and is not affected by acids. Ladies' hat-pins form a cheap but serviceable substitute. They are readily bent to any desired shape by heating. Forceps, scissors, scalpels, and many other instruments required in microscopical work do not need any special mention.

FIG. 13.—Spatula.

Bottles.—For cover-slip work and for staining on the slide dropping-bottles will be found extremely convenient. The patent T. K. pattern of 50 c.c. capacity, with flat top, is probably the best form and size.

For stains and reagents, glass- and cork-stoppered bottles of various sizes are required. The sizes most used are those containing 125, 250, 500, and 1000 c.c.

The lightning jars of half-pint and pint capacity, such as are employed for preserving fruit, can be highly recommended for holding pathological tissues after fixation. Wide-mouthed 100 c.c. bottles are useful for holding small amounts of tissue.

Incubator.—Incubators are needed for two purposes, for growing cultures of micro-organisms and for paraffin embedding. Owing to the high cost of metals the tendency at present is to replace the heavy waterjacketed copper structures of the past with simple ones made of wood and lined with asbestos. At the same time the system of keeping them at a constant temperature is steadily changing from gas to electricity because it has been found simpler and more reliable.

Storage of Microscope Slides.—Microscope slides may be stored in cabinets (the most expensive way), in boxes, or in trays. We have tried them all and use them all, but some of

them have certain advantages to which we shall call attention. Wooden cabinets with shallow drawers in which the slides lie flat can be obtained of various capacities up to those which will hold nine thousand. The important point is to have the compartments in the drawers wide enough so that an individual slide can be turned around flatwise in them without binding at the edges ($\frac{1}{4}$ inch deep, $3\frac{1}{4}$ inches wide, 15 inches long, more or less). On this account the Minot pressed tin cabinet cannot be recommended; the drawers are too shallow, the transverse ridges are a nuisance, and the lateral spacing is too narrow, so that a slide the least bit over length will not fit in it. Moreover, the drawers have a great tendency to stick in the case.

In another type of cabinet the compartments in the drawers are $1\frac{1}{4}$ inches deep, so that the slides, after the balsam is thoroughly hardened, may be stored compactly on edge in them like so many cards. The disadvantage of this method is that there is always the danger of balsam oozing out and sticking the slides together.

Several styles of boxes holding one hundred slides each are on the market. They afford the cheapest, neatest, and most convenient method of permanent storage. The green covered wooden box made by Bausch & Lomb is light in weight, the grooves are wide enough to admit the thickest slides, the alignment is true, and there is a moderate amount of play allowed for slides over length. We have found them very satisfactory.

Small wooden boxes holding twenty-five slides each are often convenient. The box marketed by Bausch & Lomb has the advantages of lightness, true alignment, and sufficient play to admit slides of various dimensions so that they will not bind. Moreover, the box has a top which cannot be confused with the bottom part; there is no danger of lifting off the wrong half.

Pressed paper trays of a capacity of twenty slides are very convenient for holding slides while the balsam is drying and before they are packed away.

EXAMINATION OF FRESH MATERIAL

Fresh tissues may be examined either in teased preparations or in sections.

Teased preparations are made by cutting out a very small bit of the tissue in question and dividing it as finely as possible, by means of two sharp, clean needles, on a slide in a drop or two of some indifferent fluid, such as the normal salt solution. Teased preparations are often made, for instance, of the heart-muscle when fatty degeneration is suspected. If the tissue is soft, the cells are easily obtained by simply scraping the cut surface with the edge of the knife.

Sections of fresh tissues can be made with a razor or with a double knife, but much the better way, at least for general diagnostic purposes, is to use frozen sections, which can be very quickly and perfectly made with the freezing microtome. The fresh sections are put into salt solution or into ordinary tap water in a glass dish large enough to permit of a slide being dipped into it, so that a section can be floated and spread out evenly on its surface. The slide is then carefully raised, the excess of fluid is wiped off, and a coverslip put on.

If it is desired to stain a section, pour over it a few drops of a ½ per cent. solution of thionin in 20 per cent. alcohol, stain for one-half to one minute or longer (over-staining does not occur), wash quickly in water, and mount *in water*. The result is a brilliant differential stain; nuclei blue or purple, collagen reddish, elastin light green.

For demonstrating amyloid either Lugol's solution of iodin or methyl-violet followed by 1 per cent. acetic acid can be used.

Sections of fresh tissue may be fixed, stained, cleared, and mounted in balsam by a slight modification of the method described for frozen sections. This modification consists in covering the section with 95 per cent. or absolute alcohol after it has been spread out evenly on the slide as described in the method referred to. The alcohol is to be dropped on the section carefully from a drop bottle, in order to avoid folding. After thirty seconds the alcohol is drained off and the section flattened

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out on the slide with blotting-paper and further treated according to the method above mentioned. If the section is not treated with alcohol before blotting, it will adhere to the blotting-paper and not to the slide.

Fresh preparations are often treated with chemicals for various purposes. Of these chemicals *acetic acid* is the most generally useful in pathological work. It shrinks the nuclei and renders their outlines more distinct. It swells connective tissue, making it more transparent, so that the elastic fibers which are unaffected stand out distinctly. It precipitates mucin and dissolves or renders invisible the albuminous granules. Its main use as a reagent for fresh tissues is to demonstrate fat and to differentiate that substance from albuminous granules.

Acetic acid is ordinarily used in a 1 to 2 per cent. aqueous solution, a few drops of which are placed at one edge of the coverslip, and then drawn beneath it by placing a piece of filter-paper on the opposite side. If in a hurry, however, stronger solutions, or even glacial acetic acid, may be used. Other reagents are of less importance, but are occasionally used.

Osmic acid is sometimes employed in a 1 per cent. aqueous solution to demonstrate fat, which it stains brown to black.

An alcohol-acetone solution of scarlet red is being used more and more for the same purpose. It stains fat orange to red.

Hydrochloric acid in a 3 to 5 per cent. solution is used to demonstrate calcification. Phosphate of lime is simply dissolved, while from carbonate of lime bubbles of carbon-dioxid (CO_2) are set free.

Indifferent Fluids.—Fresh tissues are usually examined in an isotonic salt solution—a 0.9 per cent. solution of common salt in water. It has the advantage over water that tissues do not swell up so much in it, blood-corpuscles are unaffected, and the finer structures are better preserved. A very few drops of Lugol's solution added to the stock-bottle of salt solution will be found useful in preventing the growth of mould.

Serous fluids, such as hydrocele fluid, are occasionally used. Artificial serum is made by adding 1 part of egg-albumin to 9 parts of normal salt solution.

Macerating fluids are little used in pathology. Occasionally, however, when tissues are tough, so that they cannot be readily teased apart, they are macerated in certain fluids which dissolve the substances that hold the different elements together. The reagents most commonly used are the following:

1. *Ranvier's one-third alcohol* is made by taking 1 part of 96 per cent. alcohol and 2 parts of water; twenty-four hours are usually enough.

2. Very dilute solutions of *chromic acid* are recommended— $\frac{1}{100}$ to $\frac{1}{300}$ of 1 per cent.

3. 33 *Per Cent. Caustic Potash.*—Tissues are macerated in a few minutes to one hour: they must be examined in the same fluid, because the cells are destroyed if the solution is weakened.

Examination of Fluids.—Small fragments of tissue should be picked out with forceps. If much blood is adherent, wash the tissue well in salt solution. When the cellular elements are few in number, they are obtained with a pipette, just as in urine-work, after allowing them to settle at the bottom of the glass. A centrifugal machine will be found of great service when the sediment is slight.

INJECTIONS

Injections are not much used in pathology. The process is an art that requires much patience and considerable experience. The purpose of an injection is to render vessels and vessel-walls more visible than under ordinary circumstances. Transparent, deeply colored fluid mixtures are used, which will become hard in the vessels. Some injection-masses are employed cold, others warm. The warm injection-masses contain gelatin, and are much more troublesome to use, but give much the more perfect results. For coloring the mass carmine is the best material, because it is a permanent color.

The instruments required are cannulas of various sizes and a syringe. or, better still a constant-pressure apparatus.

When a warm injection-mass is used, the bottle containing the mass must be placed in a water-bath and kept at a temperature of about 45° C. The organ or animal to be injected

must likewise be placed in a water-bath of the same temperature.

It is very important that in connecting the end of the tube carrying the injection-mass with the cannula inserted in the vessel no air-bubbles shall enter. When blood-vessels are to be injected, it is advisable to wash them out first with normal salt solution.

Cold Injection=masses.—1. *Blue Coloring Mass.*

| Soluble Berlin blue, | 1; |
| Distilled water, | 20. |

2. *Carmine Injection-mass* (Kollmann).—Dissolve 1 gram of carmine in 1 c.c. of strong ammonia plus a little water; dilute with 20 c.c. of glycerin. To this solution add 1 gram of common salt (NaCl) dissolved in 30 c.c. of glycerin. To the whole solution add an equal quantity of water.

Fischer has obtained good results by washing out the vessels in the usual way with physiological salt solution, or, better still, with a fibrin-dissolving fluid such as a freshly filtered 8 per cent. solution of nitrate or sulphate of sodium and then injecting good fresh milk. Fix the tissues for at least twenty-four hours in a 10 per cent. solution of formalin plus 2 per cent. of acetic acid.

Cut frozen sections and stain with scarlet red. Counterstain in alum-hematoxylin. Mount in glycerin. The fat can also be stained with osmium tetraoxid. The capillaries are outlined by the fat emulsion stained red or black.

Warm Injection=masses.—1. *Berlin Blue.*—Warm the solution of Berlin blue given above, and add it, with continual stirring, to an equal quantity of a warm, concentrated solution of gelatin prepared as follows: Allow clean sheets of the best French gelatin to swell up for one to two hours at room-temperature in double the quantity of water. Then dissolve them by warming gently over a water-bath. Filter the combined solution through flannel.

2. *Carmine-gelatin Mass.*—This is by all means the best injection-mass to use, because it is permanent, but it is very difficult to prepare.

Dissolve 2 to 2.5 grams of best carmine in about 15 c.c. of water, to which just enough ammonia is added, drop by drop, to effect the solution. Filter the fluid obtained, and add it, with continual stirring, to a filtered warm, concentrated solution of gelatin (prepared as above) over the water-bath. Then add acetic acid slowly until the color changes to a bright-red shade. The exact amount desired is when the solution loses its ammoniacal odor and has a peculiar sweetish aroma free from acid. Examined under the microscope, no granular precipitate of carmine should appear. If too much acetic acid has been added, so that the carmine is precipitated, the mass must be thrown away and a new lot prepared.

Organs which have been injected with a cold mass are placed directly in 80 per cent. alcohol. After a few hours they are to be cut up into pieces that are not too small. After a warm injection-mass the organ or animal is placed first in cold water to hasten the solidification of the gelatin, and then transferred to 80 per cent. alcohol.

FIXING REAGENTS

The various reagents used for the preservation of fresh tissues possess the properties of penetrating, killing, fixing, hardening, and preserving in different degrees. Of these properties "fixing" is the most important, and to a certain extent implies or includes the others. The term "fixative" has been used more particularly, perhaps, for reagents which preserve faithfully the various changes of the nucleus in karyomitosis. In a broader sense, however, it refers to the faithful preservation of any tissue-element or pathological product, and of the chemical properties peculiar to that element or product. A good fixative is a reagent that penetrates and kills tissues quickly, preserves the tissue-elements, and particularly the nuclei, faithfully in the condition in which they are at the moment when the reagent acts on them, and hardens or so affects them that they will not be altered by the various after-steps of dehydrating, embedding, staining, clearing, and

mounting. Most fixatives are mixtures of different reagents so combined that all the desirable properties may be present in as large a degree as possible.

The choice of the proper fixing reagent for a given tissue is often difficult, and must depend largely on the nature of the pathological lesions present or suspected, and on the purposes for which the tissue is preserved. The best general fixative yet devised for faithful preservation of all kinds of tissues is Zenker's fluid. It is recommended above all others after many years of constant trial.

Helly's modification of it is preferred by some and is indispensable for the preservation of certain cytoplasmic granules which are dissolved by the acetic acid in Zenker's fluid. Orth's fluid, perhaps, ranks next, but does not permit nearly so great a variety of stains to be used after it as Zenker's fluid. It has the advantage of costing much less. As a general fixative for all sorts of tissues when the main desire is to obtain reasonably faithful fixation for diagnostic purposes, formaldehyde has, to a large extent, replaced alcohol. It permits about all the chemical reactions to be performed which are possible after alcohol fixation, and has the additional advantage of preserving fat of all kinds, and especially the myelin in the sheaths of nerve-fibers.

It is strongly advised that in all important cases tissues be hardened both in Zenker's fluid and in formaldehyde: in Zenker's fluid for general histological study, and for the preservation of nuclear figures, bacteria, and fibrils of all kinds; in formaldehyde for the preservation of fat, myelin, and various substances, such as amyloid and hemosiderin, to which it may be desirable to apply chemical tests. For certain specific purposes other fixatives are sometimes required, such as alcohol for the preservation of glycogen, pigments and sodium urate crystals, and corrosive sublimate for mucus.

Tissues fixed in a solution of formaldehyde or in alcohol may remain as long as desirable in those fluids. Tissues hardened in most of the other fixatives must be transferred, after thorough washing in water, to alcohol for preservation. It is usually

recommended to pass the specimens through graded alcohols, either through 30, 60, 90, and 96 per cent., or through 50, 70, and 96 per cent., allowing them to remain from a few hours to a day in each strength. For most purposes it will be found sufficient to transfer the specimens directly from water to alcohol of 70 to 80 per cent., in which they may remain until it is desired to embed them.

Alcohol extracts chrome salts from tissues hardened in solutions of them. As these salts are precipitated in the alcohol under the action of light, it is desirable, although by no means necessary, to keep all such specimens in the dark.

It is strongly urged by some that distilled water be used in making all fixing solutions, and also that all fixatives be employed at body temperature because they will then penetrate more quickly and the tissues will, therefore, be preserved more faithfully.

Alcohol is a fair general fixative which both hardens and dehydrates tissues at the same time. As a fixing reagent formerly in much use its place is largely taken nowadays by formaldehyde. In its favor are several points. Bacteria, fibrin, various pigments, elastic fibrils, and certain cytoplasmic granules stain well after it, and it is the only fixative which preserves glycogen and allows it to be stained differentially. Its disadvantages are that it removes hemoglobin from the red blood-corpuscles, shrinks tissues more or less, and does not give them so good a consistence as some of the other fixatives. Its greatest use is as a preservative of tissues after they have been fixed and hardened by other reagents. The strength of alcohol ordinarily used in laboratories is 95 to 96 per cent. Absolute alcohol is much more expensive. Tissues hardened in either of these strengths shrink a great deal. The exposed surface becomes extremely hard, and the outer layers of the cells of tissues, like a rabbit's kidney, for example, are as shrunken and flattened as though dried in the air. It is only inside of this hard casing, where the alcohol has penetrated more slowly and has been somewhat diluted by the fluid of the tissue, that the cells are better preserved. Moreover, this extreme

hardening of the surface hinders the penetration of the alcohol into the deeper parts.

Tissue which is to be hardened in absolute or 95 per cent. alcohol should be cut into thin pieces, preferably not over $\frac{1}{2}$ cm. thick. The volume of alcohol used for hardening should be fifteen to twenty times as great as the specimen, and should be changed after three or four hours. The tissue should be kept in the upper part of the alcohol by means of absorbent cotton, or the jar may be frequently inverted and the alcohol thus kept of even strength.

The advantages of strong alcohol, 95 per cent. and absolute, are that the tissue is more quickly fixed than with weaker strength, and that at the same time it is made quite hard—a quality more necessary formerly than now when tissues are so generally embedded. Tissues hardened in strong alcohol should later be transferred to 80 per cent. alcohol for preservation, or the staining properties will gradually become impaired.

For general purposes it will be found better to place tissues at first into 80 per cent. alcohol, which should be replaced in two to four hours by 95 per cent. alcohol. In this way less shrinkage is caused and the surface of the tissues is not made so hard.

Tissues which have been fixed in Zenker's and other fluids should, after thorough washing in running water, be placed directly in 80 per cent. alcohol for further preservation. Change the alcohol occasionally as it becomes cloudy.

Formaldehyde.—The gas formaldehyde (HCOH) is soluble in water to the extent of 40 per cent. Solutions of this strength are manufactured by different commercial houses under the names of formalin, formol, and formalose. The best strength of formaldehyde to use for fixing tissues is a 4 per cent. solution; that is, 10 parts of the aqueous 40 per cent. solution, no matter what name is given to it, to 90 parts of water. Unfortunately, formic acid gradually develops in formaldehyde and exerts an injurious action on tissues preserved in it.

On this account it is advisable for most purposes to neutralize the stock solution of formaldehyde by adding carbonate of

calcium (powdered marble serves well) or lead oxide or carbonate in excess.

For certain purposes, however, it is sometimes advisable to add 5 per cent. by volume of glacial acetic acid to the ordinary solution in order to improve its fixing properties, but tissues cannot be left in the mixture. They must be transferred after twenty-four hours to the plain formaldehyde solution.

Formaldehyde penetrates very quickly. Its hardening action is not understood. It does not precipitate albuminous bodies, but makes them quite firm. It also hardens nerve-sheaths, acting toward them and red blood-corpuscles like the chrome salts. Formaldehyde is very useful for preserving gross specimens, because it gives them a rather tough, elastic consistence and preserves the normal color better than other hardening fluids, and also the transparency of many parts, such as the cornea. In general histological work formaldehyde is largely used now-a-days as a fixative in place of alcohol.

As a fixative for specimens that are to be embedded in paraffin it is not recommended unless combined with other reagents, such as bichromate of potassium in Orth's fluid, because it does not appear to harden the tissue elements sufficiently to enable them to resist the shrinking effects of prolonged exposure to alcohol and heat in the process of embedding. In frozen sections, however, prepared by the method described elsewhere, this shrinkage of the tissue elements is not apparent, probably because prolonged exposure to dehydrating, clearing, and embedding agents is avoided.

The advantages of formaldehyde are that it is comparatively cheap, can be obtained commercially in compact form, and keeps well. It fixes and hardens tissues, including red blood-corpuscles, quickly and well even in large pieces and gives them a good consistence, so that they can be cut easily on the freezing microtome or after embedding in celloidin. It permits the use of a large variety of staining methods. It also fixes and preserves fat so that this substance can be easily stained in frozen sections. In addition, it preserves myelin, and on this account is the best preliminary fixative of the central

nervous system that we have, but must be followed by a chrome salt or be combined with it.

The disadvantages of formaldehyde are that it dissolves glycogen and uric acid and biurate of sodium crystals, often changes bile concretions from a yellow to a green color, does not preserve iron and other pigments so well as alcohol, and frequently gives rise to a fine dark brown or black crystalline precipitate in the tissues. Two methods are recommended for removing the precipitate.

A. *Verocay's method:*
1. Place the sections in the following mixture for ten minutes:

    1 per cent. aqueous solution of caustic potash,   1 c.c.
    80 per cent. alcohol,   100 "

2. Wash thoroughly in at least two changes of water for five minutes.
3. Place in 80 per cent. alcohol for five minutes.
4. Return again to water.

B. *Schridde's method:*
1. Place sections in the following mixture for half an hour:

    75 per cent. alcohol,   200 c.c.
    25 per cent. solution of ammonia,   1   "

2. Wash thoroughly in water.

Formaldehyde does not of itself give tissues a sufficient consistence so that they will stand embedding in paraffin without shrinking. On this account it is best combined with a chrome salt, as in Orth's or Helly's fluid, when this method of embedding is desired.

Alcohol and Formaldehyde.—

    Alcohol (95 per cent.),   90 c.c.
    Formaldehyde (40 per cent. solution),   10 "

This combination of alcohol and formaldehyde is a most useful fixing mixture, especially for rapid diagnosis of routine surgical specimens. It fixes and dehydrates at the same time,

and yet gives better and more faithful preservation than acetone, which is often employed for the same purpose.

The following method in constant use for years in the Pathological Laboratory of the Boston City Hospital yields excellent permanent mounts. Of course, all good tissues are also fixed in Zenker's fluid, cut after paraffin embedding, and stained with eosin and methylene-blue and by any other method that seems desirable.

1. Fix thin sections of tissue, 2 to 4 mm. thick, 1–4 hours.
2. 95 per cent. alcohol,   1–2   "
3. Absolute alcohol,   1–2   "
4. Absolute alcohol and ether, equal parts,   1–3   "
5. Thick celloidin,   6–12   "
6. Chloroform,   ¼–1 hour
7. 80 per cent. alcohol,   a few minutes to 1   "

8. Cut and stain in alum hematoxylin and eosin, dehydrate in 95 per cent. alcohol, clear in oleum origani cretici, and mount in xylol colophonium. Soaking the tissues in thin celloidin is not ordinarily necessary because they contain enough alcohol and ether to dilute the thick celloidin immediately around them.

Corrosive sublimate is a most useful fixing reagent, but is best employed in combination with a chrome salt, as in Zenker's and Helly's fluids. Its great disadvantage when used alone is that it causes serious shrinkage of the cells. A second disadvantage, which attends its use under all conditions, is that it gives rise to a crystalline precipitate of mercuric oxide. This precipitate can be removed from the tissues by means of iodin, which forms a colorless soluble compound.

Do not add iodin to the alcohol in which the tissues are preserved, because prolonged treatment with iodin exerts an injurious effect on the staining properties of the cells. Embed the tissues and cut sections without removing the precipitate, and then treat the sections, just before staining, with Lugol's solution of iodin for ten to twenty minutes, followed by alcohol to remove the iodin.

Inasmuch as prolonged action with alcohol is frequently necessary in order to remove the iodin, it is often better to use a 5 per cent. aqueous solution of sodium hyposulphite for this purpose instead of alcohol, because it acts almost instantaneously and is then itself easily removed by thorough washing in water.

It is only fair to state that many workers prefer to add iodin to the alcohol in the stock jar of tissue until the color no longer disappears in order to remove the precipitate before embedding and cutting sections.

The directions for the use of corrosive sublimate are as follows: Use a saturated aqueous solution made with the aid of heat.

The addition of 5 per cent. of glacial acetic acid is usually advisable. 1. Harden thin pieces of tissue (2 to 5 mm.) for six to twenty-four hours. 2. Wash in running water twenty-four hours. 3. Preserve in 80 per cent. alcohol.

Tissues hardened in corrosive stain quickly and brilliantly in nearly all staining solutions. It is the only fixative after which the Heidenhain-Biondi triple stain gives good results.

Giemsa's Corrosive Sublimate=alcohol Fixative.—Saturated aqueous solution of corrosive sublimate, 2 parts; Absolute alcohol, 1 part.

It requires at least forty-eight hours, and is to be renewed after twenty-four hours. The tissue may remain as long as three months in the solution without disadvantage if evaporation is prevented.

This fixative is the one usually recommended for tissues which are to be stained by Giemsa's method, but S. B. Wolbach has shown that excellent results, but with a reversal of the color effect, may be obtained after fixation in Zenker's fluid, and the tissue preservation is much better.

Zenker's Fluid.—

Bichromate of potassium,	2.5 grams;
Corrosive sublimate,	5 to 8 "
Water,	ad 100 c.c.;
Glacial acetic acid,	5 "

Dissolve the corrosive sublimate and the bichromate of potassium in the water with the aid of heat.

Do not add the acetic acid to the stock solution, but only in the proper proportion to the part taken for hardening pieces of tissue, because the acid evaporates so readily, and also produces changes in the chrome salt.

Zenker's fluid was originally Müller's fluid plus 5 per cent. of corrosive sublimate and 5 per cent. of glacial acetic acid, but the sulphate of sodium is usually omitted nowadays because it is generally agreed that it serves no useful purpose. Personally we have always added corrosive sublimate in excess (7 to 8 grams to each 100 c.c. of fluid) so as to have the solution saturated with it.

Directions for Use.—1. Fix tissues in the solution twelve to twenty-four hours.

2. Wash in running water twelve to twenty-four hours.

3. Preserve in 80 per cent. alcohol until used.

Tissues float at first in this solution, which penetrates fairly quickly.

Zenker preparations stain slowly but beautifully in alum-hematoxylin. The most brilliant results, however, are obtained by staining with eosin, followed by Unna's alkaline methylene-blue solution. Excellent results are also obtained after staining in phosphotungstic-acid hematoxylin, and by the anilin-blue method. They bring out fibrin and various kinds of fibrils in addition to nuclear details.

When sections of Zenker-fixed tissues which have been kept for a long time are stained with alum-hematoxylin the places where the crystalline deposit was present are colored deep blue and thus disfigure the specimen. The only way found so far to prevent this staining is to soak the sections for several weeks in acid alcohol before staining them. This treatment causes no injury to the tissues, but does, as a rule, prevent the disfiguring stains from appearing. On the other hand, the method will also remove certain pigments from the sections and, therefore, cannot always be used.

Helly's fluid is a slight modification of Zenker's fluid: the glacial acetic acid is replaced by 5, occasionally 10, per cent. of strong formaldehyde added just before the mixture is used. For certain purposes, such as fixing the cytoplasmic granules in the islet cells of the pancreas, the formaldehyde should be carefully neutralized.

Bichromate of potassium,	2.5 gr.;
Corrosive sublimate,	5 to 8 "
Water,	100 c.c.;
Formaldehyde (40 per cent. solution),	5 to 10 "

1. Fix tissues in the fluid for twelve to twenty-four hours.
2. Wash in running water twelve to twenty-four hours.
3. Transfer to 80 per cent. alcohol.

Chrome Salts.—Chromic acid is rarely used nowadays except in Flemming's solution. The chrome salts are employed instead, especially the bichromate of potassium, which enters into several well-known fixing solutions. It penetrates slowly and is a poor fixative of neuclear material, but is the best of all known hardening reagents. On this account tissues fixed in solutions containing it stand paraffin embedding with little or no shrinkage. Bichromate of potassium has been used so long in the solution known as Müller fluid that the latter solution is regarded as practically synonymous with it.

Müller's Fluid.—

Bichromate of potassium,	2 to 2.5 grams;
Sulphate of sodium,	1 gram;
Water,	100 c.c.

Harden tissues six to eight weeks. Change the fluid daily during the first week; once a week thereafter. Ordinary tissues are then washed in running water overnight before being place in alcohol. Nervous tissue is transferred directly from the fluid to the alcohol.

This famous hardening solution is rapidly giving way to better fixatives. It hardens tissues slowly, evenly, and with little or no shrinkage, but it is a poor nuclear fixative, and does

not encourage any great variety of stains. The sulphate of sodium seems to serve absolutely no function. For ordinary tissues it is being replaced by Zenker's, Helly's, and Orth's fluids, all of which fix very quickly, besides having all its good qualities. For nervous tissues formaldehyde followed by other solutions of the chrome salts is a great deal quicker and better.

Tellyesniczky has recommended the following mixture, which has met with considerable favor, and which may be regarded as an improved Müller's fluid:

Bichromate of potassium,	3 parts;
Water,	100 "
Glacial acetic acid,	5 "

Fix thin sections for one or two days; thicker sections longer. Wash out thoroughly in running water. Dehydrate in graded alcohols.

Orth's Fluid.—This is a general fixative consisting of the well-known Müller's fluid plus 4 per cent. of formaldehyde:

Bichromate of potassium,	2 to 2.5;
Water,	100;
Formaldehyde (40 per cent. solution),	10.

The formaldehyde should be added only at the time of using, for in two days the solution becomes darker, and by the fourth day a crystalline deposit begins to take place. As fixation is ordinarily complete in three to four days, this deposit does not matter. The tissue should not be over 1 cm. in thickness. Small pieces, $\frac{1}{2}$ to $\frac{1}{3}$ cm. in thickness, can be readily hardened in the incubator in three hours. The specimens should be washed thoroughly in running water six to twenty-four hours before placing in 80 per cent. alcohol.

The method is particularly recommended for mitosis, red blood-corpuscles, bone, and colloid material (in cystomata, etc.), as it gives a very good consistence to the tissues, but the histological detail is not so good as after Zenker's fluid. The addition of 5 per cent. of acetic acid would unquestionably improve it.

Regaud's Fluid.—

3 per cent. aqueous solution of bichromate of potassium	4 parts;
Formaline	1 part.

Recommended for fixation of tissues containing Rickettsia and for mitochondria. Directions for use as for Zenker's fluid.

Osmic Acid.—The tetroxide of osmium, commonly known as osmic acid, is a fixing reagent of considerable value, particularly for the demonstration of fat, but penetrates tissues poorly. On this account it is generally used in combination with other reagents some of which seem to increase its power of penetration.

Flemming's Solution.—

Osmic acid, 2 per cent. aqueous solution,	4;
Chromic acid, 1 per cent. aqueous solution.	15;
Glacial acetic acid,	1.

1. Fix in the solution one to three days. 2. Wash in running water six to twenty-four hours. 3. Alcohol, 80 per cent.

It is best to keep the osmic acid in a 2 per cent. solution and the chromic acid in a 1 per cent. solution. The mixture can then be quickly made up fresh at the time it is needed. The best stains after hardening in Flemming are Babes' safranin, aniline-methyl-violet, and carbol-fuchsin.

Pieces of tissue for hardening in Flemming's solution should not be over 2 mm. in thickness, because it has very slight penetrating properties.

Marchi's Fluid.—

Müller's fluid,	2 parts;
Osmic acid, 1 per cent. aqueous solution,	1 part.

Place small pieces of tissue in the mixture for five to eight days, wash thoroughly in running water, and harden in alcohol. For its application to degenerated nerve-fibers see page 158.

Hermann's Solution.—

Osmic acid, 2 per cent. aqueous solution,	4;
Platinic chlorid, 1 per cent. aqueous solution,	15;
Glacial acetic acid.	1.

This modification of Flemming's solution is perhaps an even better fixative than the model on which it is based, but is more expensive. It should be employed in the same manner.

Pianese's Solution.—

Chlorid of platinum and sodium, (platinic) 1 per cent.
  aqueous solution   15 c.c.;
Chromic acid, $\frac{1}{4}$ per cent. aqueous solution,   5 "
Osmic acid, 2 per cent. aqueous solution,   5 "
Formic acid, C. P.,   1 drop.

Fix small pieces of tissue, not over 2 mm. thick, in the solution for thirty-six hours. Wash in running water for twelve hours, then 80 per cent. alcohol. Stain paraffin sections by Pianese's special methods (see p. 82).

This fixative and the special staining methods are particularly recommended for the study of karyomitosis and of the so-called cancer bodies.

Boiling.—Boiling precipitates the soluble albumin in tissues as a granular material which can be readily recognized. The method is used particularly for the demonstration of albumin in renal diseases and in edema of the lungs. By means of boiling the quickest permanent mounts of tissues can be obtained. The method is not advocated on account of the shrinkage caused by the heat, but will sometimes be found useful. Occasionally 10 per cent. or even 40 per cent. formaldehyde is employed instead of water.

Small pieces of tissue not over 1.5 cm. in diameter should be dropped into the boiling water for one-half to two minutes; cool quickly in cold water, and make frozen sections, or put into 80 per cent. alcohol. Any stain may be used; methylene-blue will be found excellent.

DECALCIFICATION

Tissues which are to be decalcified should be sawn with a fine hair-saw into thin slices, so that they will decalcify quickly. It is usually desirable to saw the tissue into pieces of proper size for embedding in celloidin. Very dense bone ought not to be over 2 or 3 mm. thick; softer tissues do not need to be thinner

4

than 4 to 6 mm. In cutting sections after decalcifying and embedding it is necessary to throw away the first-half dozen sections or so, because the tissue is so lacerated to a slight depth by the movement of small fragments of bone in the process of sawing as to be useless for microscopic purposes. The extent of the decalcification may be tested at any time by thrusting a needle into the tissue, but it is best to avoid such a test because, of course, it tends to produce injury to the tissue.

The following steps in the decalcification of tissues must be carefully borne in mind.

1. The tissues must first be thoroughly hardened. The most useful reagents for this purpose are alcohol, the alcohol-formaline mixture and Zenker's and Orth's fluids. After the two latter reagents the tissues must have been washed thoroughly in water and placed in alcohol for at least twenty-four hours. They will then be ready for decalcification.

2. The decalcifying fluid must be used in large amounts, and, if necessary, be frequently changed. Decalcification should never be prolonged beyond four days if possible; twenty-four to forty-eight hours are better.

3. After decalcification the tissues must be thoroughly washed in running water for twenty-four hours to get rid of every trace of the acid.

4. The tissues finally must be hardened again in alcohol.

Of the various agents used for decalcifying bone, nitric, hydrochloric, chromic, picric, trichloracetic acids, etc., the most important is nitric acid. It acts quickly, without swelling the tissues or attacking injuriously the tissue-elements, and does not interfere to any marked degree with any subsequent staining process. Red blood-corpuscles will be found uninjured in tissues hardened in Zenker's fluid even after remaining four days in 5 per cent. nitric acid. This acid is used in dilute solution alone or in combination with phloroglucin.

Directions for Using Nitric Acid.—1. Decalcify in large quantities of a 5 per cent. aqueous solution of nitric acid. changing the solution every day for one to four days. 2. Wash twenty-four hours in running water to remove every trace

of acid. 3. Harden in 80 per cent., and then 95 per cent. alcohol. Embed in celloidin. According to Schaffer, it is best to transfer the tissue directly from the nitric acid to a 5 per cent. solution of alum for twenty-four hours before placing in running water, so as to avoid any possibility of the tissue swelling, but this step hardly seems necessary.

Schridde recommends highly the following strong solution for rapid decalcification:

Formaldehyde (strong solution), ·	10 c.c.;
Distilled water,	90 "
Nitric acid,	20 "

Use at body temperature; two to three hours are usually sufficient, but tissues may remain in the solution for twenty-four to forty-eight hours. Wash for twelve to twenty-four hours in running water and then transfer to 80 per cent. alcohol.

Phloroglucin and Nitric Acid.—Phloroglucin is not a decalcifying agent, but is added to nitric acid to protect the tissues while allowing a stronger solution of the acid to be used than would otherwise be possible. The solution is prepared by dissolving 1 gram of phloroglucin in 10 c.c. of nitric acid. Solution takes place quickly, with generation of considerable heat. The fluid is reddish brown at first, but becomes light yellow in the course of twenty-four hours. Dilute with 100 c.c. of a 10 per cent. solution of nitric acid. This gives nearly a 20 per cent. solution of nitric acid. The process of decalcification in this fluid is extremely rapid; a few hours only, as a rule, are required. It is not advisable to dilute the solution by the simple addition of water, but by the use of less acid, because the phloroglucin must be present to the amount of 1 per cent. or it will not protect the tissues so well.

The following slower-acting solution may be found useful:

Phloroglucin,	1;
Nitric acid,	5;
Alcohol,	70;
Water,	30.

A rather deep single stain with alum-hematoxylin (either aqueous solution or Delafield's) will usually be found to give the best results with tissues decalcified with nitric acid. It is very important to leave the sections after staining in a large dish of water overnight, otherwise the stain will not be so sharp and clear.

Sulphurous Acid.—A saturated solution—about 5 per cent.—is used. It works very quickly and causes little swelling. The tissues should be carefully washed out in running water as after nitric acid. The stock solution rapidly grows weak through evaporation if the bottle in which it comes is not kept tightly corked.

Trichloracetic Acid.—A 5 per cent. solution of this acid has lately been recommended for the decalcification of bone and teeth. It acts more slowly than nitric acid, and seems to possess no advantages over it. Tissues must be washed out in running water, as after nitric acid.

FROZEN SECTIONS

In making frozen sections with a simple microtome such as as described on page 21 the following directions may be helpful. It should be emphasized that satisfactory sections are much more easily made with the more complicated machines, especially those with automatic feed mechanism.

The knife must be sharp and free from nicks. It must have a chisel edge, as shown in Fig. 2. It should be sharpened by grinding on a hone, and afterward by thoroughly stropping on a razor-strop. Frequent stropping is just as necessary as in the case of the ordinary microtome knife.

In cutting, grasp the knife by the thick wooden handle so that the end presses against the ball of the thumb and the palm of the hand, while the dorsum of the hand is uppermost; then, with the wrist flexed and held against the chest, apply the edge of the knife to the glass ways of the microtome in such a manner that the edge, bevel side downward, is at right angles to the direction of the ways and the long axis of the knife at an angle of 45 degrees to their surface; now, holding

the knife and wrist rigidly in the positions just indicated, push the cutting-edge quickly forward along the ways through the specimen by moving the body forward from the waist, in the mean while pressing the cutting-edge steadily downward upon the ways with constant force. Thus a strong constant downward pressure of the edge upon the ways is maintained, and at the same time great steadiness and power are given to the cutting stroke, which are conditions that are very important for obtaining thin sections. With the fingers of the other hand manipulating the wheel of the microtome screw, a number of sections should be cut in quick succession in the manner indicated without changing the angle of the knife or the position of the hand and wrist above described, the edge of the knife on the backward movement being lifted from the ways only enough to clear the cut surface of the specimen. The sections will usually adhere to the knife, and a number of them may be allowed to collect thereon. They are removed from the knife by immersing it in water, in which they will float and flatten out, no matter how much wrinkled and compressed upon the knife they have been. The cutting of a number of sections in quick succession without pausing to remove each section from the knife seems to be necessary for obtaining the thinnest sections.

The consistence of the frozen tissue is important. The specimen immediately after freezing will usually be too hard to cut without yielding sections that break over the edge of the knife, and are, therefore, to be rejected. If this happens, wait a few seconds and thereafter cut a section or two at short intervals until the specimen is found to have a consistence yielding satisfactory sections, whereupon a number of sections should be cut in quick succession as above described.

The piece of tissue from which the sections are to be cut should be not thicker than 5 mm., and a little water should be placed under it on the freezing box to bind it thereon.

Before staining, the section should be attached to the slide to avoid distortion and facilitate handling. Two good methods of doing this are given here.

Aniline=clove Oil Method.— Coat the slide with a thick layer of Mayer's albumin fixative and float the section on to it, spreading smoothly. Next wipe away most of the fluid from around the section and press the section on to the slide with smooth blotting paper. Then without allowing the section to dry out, cover it with a mixture of equal parts of aniline and clove oil, and immediately rinse off the mixture with 95 per cent. alcohol. After immersing in water to remove the alcohol, the section, thus attached to the slide, is ready for staining and mounting.

In spreading the section on the slide too long immersion of the slide in the water may wash off the fixative and the section will not stick. This very rarely happens after a little practice.

Celloidin Method.—Float the section on to the slide from water, spread it out smoothly, and press or blot it upon the slide with fine fiber blotting or filter paper. Next cover it with alcohol for about half a minute and blot it again. Then flow over the section and adjacent part of the slide a dilute solution of celloidin in equal parts of absolute alcohol and ether, drain off excess fluid at once, blow the breath briskly on the section, and immediately immerse the slide in water for a few seconds to harden the celloidin. Thus the section is attached to the slide by a thin film of celloidin and may be stained by any of the usual methods, for the celloidin does not prevent the penetration of stains and does not interfere with the visibility of the section.

The solution of celloidin should be sufficiently dilute to flow readily and not to form too thick a film.

Drying of the section at any stage should be avoided by proceeding with quickness.

If the tissue was not properly fixed before sectioning, or if it contains mucoid material, the section may stick to the blotter. This may be prevented by treating the section with alcohol before blotting.

For clearing, after staining and dehydrating, Oil of Origanum or Red Oil of Thyme (which is much cheaper) are recommended.

Embedding Method for Frozen Sections (Wright).— This method permits the making, staining and permanent

mounting of coherent frozen sections of fragmented tissue, such as curettings, and of flimsy or loose textured tissue, with minimum time and manipulation.

The embedding mass is a 10 per cent. "solution" of gelatin in distilled water, with which, while warm and fluid, 0.5 per cent. carbolic acid has been mixed. The mass should not be heated more than is necessary for thorough solution. It keeps well in a stoppered flask.

The tissue should be already fixed, preferably in 4 per cent. aqueous solution of formaldehyde (10 per cent. formaline).

To embed the tissue, the mass is liquefied by heat, without boiling, a small "pool" poured on an ordinary glass slide or similar object which may be conveniently handled, and the tissue, after being dried with a towel, is immersed and arranged therein. Then the "pool" is allowed to solidify in a cool place or in the ice box for two hours or longer, after which a "block" is cut and trimmed from it; and from this "block" frozen sections are made as from a single piece of tissue.

The mass enclosing the tissue should be kept from drying out, if it be not sectioned after two hours. This may be done by placing it and the slide on which it has solidified in a closed bottle, along with so much of a 4 per cent. aqueous solution of formaldehyde as will reach up to the mass without covering it.

The sections, having been transferred from the knife to water, are to be stained, dehydrated, cleared and mounted, after being attached to the slide. The attachment to the slide is desirable for facility in handling, and is necessary to prevent curling and shriveling during dehydration through shrinking of the gelatin. They are attached to the slide by the following procedure: Coat the slide with a thick layer of Mayer's albumin fixative and float the section on to it, spreading smoothly. Remove excess of fluid from around the section, cover with a piece of thin cigarette paper, and blot with blotting paper until the cigarette paper is partly dry. Then cover the cigarette paper with a mixture of equal parts of Aniline and Clove Oil for a few seconds; drain off the oil and peel off the cigarette paper from the slide. The section adheres to the

slide, which is then washed with 95 per cent. alcohol to remove the oil and immersed in water, when the section, thus attached to the slide, is ready for staining, dehydrating, clearing and mounting.

CELLOIDIN AND PARAFFIN EMBEDDING PROC= ESSES

· Sections of hardened tissues can be cut with a razor by hand, or with a microtome knife after fastening the specimen in the microtome clamp either directly or between pieces of amyloid liver. Fair sections of firm tissues can often be obtained in this way. Thinner sections can be got by means of the freezing microtome, but these methods are all open to the objection that unless the tissue is very cohesive, portions of it are likely to fall out of the sections.

The best results would, therefore, naturally be expected from some embedding process, employing a substance to infiltrate the tissues thoroughly and to hold the different parts in proper relative position even in the thinnest sections.

The two substances in common use for this purpose are celloidin and paraffin. Each has its advantages and disadvantages. Neither can be employed in pathological histology to the exclusion of the other. Paraffin affords the thinner sections, but they must be small if the best results are desired, and cannot be properly handled except when fastened to the slide. Hard tissues, like muscle, and tissues of varying consistency, like skin, are cut with considerable difficulty by the paraffin method. Staining is rather simpler than after embedding in celloidin.

On the other hand, tissues of almost any consistency or size can be cut by the celloidin method, which is also capable of furnishing very thin sections.

Both methods of embedding should be learned and used. Celloidin sections are especially good for general work, for studying the extent and relations of pathological processes, and for much of the finer histological work. Paraffin sections

are better for the finest details of processes—for special work on special tissues.

Celloidin.—Schering's celloidin has in the past been the best preparation of gun-cotton (pyroxylin) to use. It keeps well, dissolves somewhat slowly, and gives a fairly transparent embedding mass, which is firm and tough, so that very thin sections can be cut. Mallinckrodt's Purified Pyroxylin, recently put on the market, seems to give equally good results.

Embedding in Celloidin.—The process consists in soaking the tissues for twenty-four hours to a number of days in two different solutions of celloidin. The two solutions are spoken of as thin and thick celloidin. To make thick celloidin 30 grams of the dry celloidin are dissolved in 200 to 250 c.c. of a mixture of equal parts of ether and absolute alcohol. Diluted with an equal amount of the ether-and-alcohol mixture, it forms thin celloidin.

The steps of the embedding process are as follows: Pieces of tissue which have been properly fixed and finally preserved in 80 per cent. alcohol are first to be cut up with intelligence. They should rarely be over 2 to 4 mm. thick; for most purposes 2 mm. will be found sufficient. Pieces of this thickness will furnish a hundred sections or more, will embed more quickly than larger masses, and will be more rigid when mounted on a block. They should never be broader or longer than is necessary to show the whole of the process under study. Very thin celloidin sections cannot usually be obtained with tissues over 1.5 to 2 cm. square, and smaller dimensions are preferable. Beginners usually embed larger pieces than are necessary.

The trimmed pieces of tissue are first hardened and dehydrated for twenty-four hours in 95 per cent. alcohol, followed by twenty-four hours in absolute alcohol; then soaked in equal parts of absolute alcohol and ether for the same length of time to prepare them for the thin celloidin. In the latter they remain at least twenty-four hours, preferably for a number of days, if at all thick, for in this solution occurs most of the infiltration with celloidin. Finally, the pieces are soaked twenty-four hours or more in the thick celloidin. They are

then mounted on blocks of vulcanized fiber, placed in chloro-
form for one or two hours, and then transferred to 80 per cent.
alcohol.

Briefly summed up, the steps of embedding in celloidin are
as follows:

1. 95 per cent. alcohol, twenty-four hours.
2. Absolute alcohol, twenty-four hours.
3. Ether and absolute alcohol, equal parts, twenty-four hours.
4. Thin celloidin, twenty-four hours to one or more weeks.
5. Thick celloidin, twenty-four hours to one or more weeks.
6. Mount on blocks of vulcanized fiber.
7. Harden celloidin in chloroform for one or two hours,
followed by 80 per cent. alcohol.

Instead of mounting directly from the thick celloidin, it is
often advisable to allow the celloidin to evaporate until a
firm mass is obtained. This is particularly true when very
loose tissues are to be embedded.

The simplest method is to place the pieces of tissue, which
have been soaking in thick celloidin, in proper position in a
glass dish and pour thick celloidin over them. The dish is
then covered, but not too tightly, and the ether is allowed to
evaporate for one or more days until the proper consistency
of celloidin is reached, so that it can be cut out in blocks
enclosing the specimens. If the ether evaporates too rapidly,
place a large dish or a bell-jar over the covered dish. Mount
the blocks, after they have been cut out and trimmed, by
dipping the bases in thick celloidin and then pressing them on
to blocks of vulcanized fiber.

Place them in chloroform for one or two hours and then
transfer to 80 per cent. alcohol.

After the celloidin mounts have been in 80 per cent. alcohol
for one to several hours, the celloidin is of the proper consistence
for cutting. It is best to take a sharp knife or an old razor
and trim the top of the celloidin down to where the first good
section of the specimen can be cut; this will save considerable
wear on the microtome knife.

In cutting, the microtome knife should be fastened very obliquely, so that as much as the edge of the knife as possible shall be used in making each section. The surface of the knife should be kept well wet with 80 per cent. alcohol, preferably from an overhanging drop-bottle.

If the sections curl, as often happens when they are thin, they are best flattened by unrolling them on to the surface of the knife with a camel's hair brush just before the last edge of celloidin is cut through, as this serves to keep them fixed in place during the process. This method can be used when the simple transferring of sections from alcohol to water is not sufficient to uncurl them.

Celloidin sections can be stained by nearly all methods, without the necessity of removing the celloidin. When necessary, however, the celloidin is readily removed by placing the sections from absolute alcohol in oil of cloves or in the alcohol-and-ether mixture for five or ten minutes, and then passing them back through absolute into ordinary alcohol.

To Attach Celloidin Sections to the Slide.—A celloidin section can be fairly well attached to a slide by transferring it from water to a slide freshly washed in alcohol and dried with a cloth. The section is then to be firmly blotted with filter-paper so as to apply it closely to the slide and to remove all wrinkles. It should not be allowed to dry. A section treated in this way will ordinarily stand considerable manipulation without becoming loose.

Celloidin sections can be more securely attached by transferring them from 95 per cent. alcohol to clean slides and pouring over them ether-vapor from a bottle half full of ether. With a little practice sections can be fastened in a few seconds. Follow slowly along the edge of the celloidin, and the frills in it will soften down. Then wash the specimen with 80 per cent. alcohol to harden the celloidin.

Another excellent method is that described for fixing frozen sections to the slide (see page 54).

Paraffin.—Excellent paraffin, melting at 125° F. (51.6° C.), can be obtained, when bought in large quantities, for about

eight cents a pound, from the regular dealers in paraffin, and can be used at all seasons of the year.   (E. F. King & Co., Boston, can be recommended.)   We have never found the more expensive sorts recommended by dealers in laboratory supplies necessary.

Embedding in Paraffin.—Paraffin embedding is particularly useful when very thin sections are desired.   To obtain the best results the pieces of tissue should be small, soft, and of uniform consistence.   In pathological work it is much better to cut the sections and to stain them after they are fastened to the slide than to stain in the mass beforehand, because then a variety of stains may be used.   A complete or perfect series is not so important as in embryology, but with a little care can be obtained.

The first step in the preparation of hardened tissues for the paraffin bath is to cut them into thin, square or rectangular pieces, not over 2 cm. square, perhaps, for the best results, and not over 2 to 3 mm. thick.   It should be stated, however, that with proper skill, a heavy, sharp knife, and a rigid microtome very thin paraffin sections can be obtained with tissues measuring 4 × 3 cm.   The pieces of tissue are then thoroughly dehydrated by soaking first in 95 per cent. and then in absolute alcohol.   From alcohol they are put in some substance, such as chloroform or oil of cedar, which has the property of mixing with alcohol and of dissolving paraffin.   From the chloroform they are transferred to a saturated solution of paraffin in chloroform, and then passed through two separate baths of the melted paraffin to get rid of every trace of the chloroform.   If oil of cedar is used, the specimens are transferred directly from it into the melted paraffin, or they may be placed first for half an hour or so in chloroform or xylol to get rid of the oil of cedar.   This procedure enables one to make use, for certain dense tissues, such as the skin, of the better penetrating powers of the oil, and yet avoid carrying it into the paraffin bath.

One advantage of the chloroform method is that the duration in the hot paraffin, the objectionable feature of the paraffin method, is shortened, because the tissues are already somewhat

infiltrated with paraffin. Another advantage is that the paraffin bath purifies itself, because the chloroform rapidly evaporates. When oil of cedar is used, the paraffin must be renewed frequently.

Benzene (benzol) is preferred by some workers to chloroform or xylol and can be highly recommended. It clears quickly, renders the tissues more transparent than the other reagents do, and evaporates rapidly from the paraffin bath.

The methods of embedding in paraffin are briefly stated as follows:

Method No. 1.

1. 95 per cent. alcohol,   6–24 hours.
2. Absolute alcohol,   6–24   "
3. Chloroform,   6–24   "
4. Chloroform saturated with paraffin,   6–24   "
5. Paraffin bath, two changes,   2–4   "
6. Embed and cool quickly in cold water.

Method No. 2.

1. 95 per cent. alcohol,   6–24 hours.
2. Absolute alcohol,   6–24   "
3. Oil of cedar, two changes,   6–24   "
4. Paraffin, three changes,   2– 8   "
     until no odor of oil of cedar,
5. Embed and cool quickly in cold water.

Method No. 3.

1. Acetone,   ½– 2 hours.
2. Benzene,   15–30 minutes.
3. Paraffin,   30–90   "

This method is recommended when there is great haste. We are not sure that it does not shrink the tissue more than the other methods. The quantity of acetone used should be at least twenty-five times the volume of the tissue. With larger amounts of tissue the acetone should be changed after thirty minutes or an hour, and a longer exposure to the acetone and paraffin may be necessary.

Method No. 4.

The following method is recommended for certain brittle tissues, such as guinea-pigs' livers, which are difficult to section after fixation in Zenker's fluid:

1. Transfer tissue from 80 per cent. alcohol to 95 per cent. alcohol for 3–12 hours.
2. Absolute alcohol, 12 hours.
3. Absolute alcohol and xylol, equal parts, 12 hours.
4. Oil of cedar, two changes, 48 hours.
5. Blot: xylol five minutes, two changes, $2\frac{1}{2}$ minutes each.
6. Paraffin oven, four changes in all, 4 hours.

For embedding paraffin specimens metallic boxes can be used, or forms made round or square from strips of sheet lead or tin. Many prefer paper boxes, which can be made easily of any size desired from stiff writing-paper.

Melted paraffin is poured into the paper box to the depth of about 1 cm. The pieces of tissue are then placed in the box with that side down from which sections are preferred. When all the pieces are arranged in order with about half a centimeter or more between them, the box is placed on the surface of a large dish of cold water, on which it floats, so that the paraffin may cool quickly without crystallizing. Sometimes it is advisable to set the paper box with the specimens in it in the paraffin oven for a short while, so as to get rid of any bubbles carried in by the specimens. After the paraffin has hardened, the paper is removed and the paraffin is divided up according to the pieces in it. One of the blocks is then fastened to the object-holder by heating the latter in a flame until it will just melt the paraffin when the block is held in proper position against it. The holder is then quickly cooled in cold water.

The upper surface of the paraffin should now be shaved down to the specimen. The four sides are to be carefully trimmed; the upper and lower surfaces should be parallel and not cut too close to the specimen, otherwise the sections will not adhere to each other; the lateral surfaces should, as a rule, be cut close to the tissue, especially if very thin sections are

desired, because if a rim of paraffin is left it is likely to cause wrinkling of the sections. The holder is finally carefully adjusted in the paraffin microtome.

To get good sections which will adhere to each other and form a ribbon the temperature of the room must be regulated to suit the degree of hardness of the paraffin used. An open window will often make all the difference needed to obtain good results. The harder the paraffin the warmer the room must be. The temperature can be raised by burning a Bunsen flame near the microtome or lowered by the presence of a lump of ice. It will often be found advantageous to dip the holder and paraffin block into ice water or to rest a small bag containing cracked ice on the block and knife for a few minutes just before cutting.

The ribbons of sections as cut, usually a slideful, are laid on the surface of a large dish of warm water at about 44° C., and if necessary gently stretched so as to remove all wrinkles. Paint the surface of a slide with a thin layer of Mayer's glycerin-albumin mixture, wipe off all excess with a towel so that only a faint layer is left, dip the slide under the sections, arrange them in order, lift the slide, and drain off the water. The slide is then placed in a slanting position until dry, when it is put in the incubator for two to twelve hours at a temperature of about 54° C. This process attaches the sections firmly to the slide.

To get rid of the paraffin in the sections they are treated with two or three changes of xylol, and then with absolute followed by 95 per cent. alcohol.

If for any reason the celloidin-and-oil-of-cloves mixture is used for attaching the sections to the slide, the paraffin is removed by means of xylol, followed by origanum or bergamot oil, and finally by 95 per cent. alcohol, because absolute alcohol will dissolve the celloidin.

Mayer's glycerin=albumin mixture for attaching paraffin sections to slides is composed of equal parts of the white of egg and of glycerin. The mixture should be thoroughly beaten and then filtered, or after standing for some time can be decanted. Add 1 per cent. of sodium salicylate to prevent decomposition. Egg-albumin is dissolved by acids and alkalies, so

that when such reagents are to be used the sections are best attached to the slide by some other substance. For this purpose *Schällibaum's solution*, of celloidin 1 part in 3 or 4 parts of oil of cloves, is often useful. Cover the slide with a thin layer of the solution. Arrange the sections in order on the slide and place it in the thermostat at 54° to 60° C. for several hours, or heat for a few seconds to half a minute over the flame until the oil of cloves runs together in drops. After cooling, remove the paraffin with xylol, pass through origanum oil to 95 per cent. alcohol, and proceed as with other paraffin sections.

SERIAL SECTIONS

By the Celloidin Method.—1. With a little care perfect serial sections can be made by the following method, and each slide of sections can be stained in whatever way seems best. The specimen is embedded, mounted on vulcanized fiber, and hardened in 80 per cent. alcohol in the usual way. In cutting moisten the microtome knife with 95 per cent. alcohol. As the sections are cut they are drawn up on the surface of the knife and arranged in regular order by means of a camel's-hair brush until a slideful is ready. They are then drawn on a clean and numbered slide held against the back of the knife. After being carefully arranged the sections are fastened to the slide by means of ether-vapor (see p. 59) poured over them from a half-full bottle. Care must be taken that every edge of the celloidin is fully softened down. The slides are then placed in a jar of 80 per cent. alcohol to be stained at leisure.

2. Another method, often convenient where the stain is of little importance, is as follows: The tissue is stained, in bulk, in alum-cochineal or some other staining fluid that will penetrate, and then embedded in celloidin in the usual way. After being mounted on vulcanized fiber the specimen is hardened in chloroform instead of in 80 per cent. alcohol. From the chloroform the specimen is transferred to oil of thyme. After it is thoroughly penetrated by the latter it is ready to be cut. The knife is to be moistened with oil of thyme. The sections as

cut are arranged on the knife, and then transferred to slides placed against the back of the knife. The slides covered with sections can be placed under a bell-jar as fast as they are ready until all are cut, because the oil of thyme evaporates slowly. Balsam and cover-slips can be added after the cutting is finished.

3. Darkschewitsch has recently proposed a comparatively simple method for preparing a series of celloidin sections. Take a glass cylinder without a neck, of about the diameter of the specimen to be cut. Prepare a series of circles of filter-paper cut of a size just to fit the bottle, number in order, and wet them with alcohol. Each section is removed from the microtome knife by pressing one of the paper circles upon it and drawing it off. The paper is then inverted so that the section is uppermost, and deposited in proper order in the bottle, where the series forms a column, each section resting upon a numbered paper. The sections can be kept indefinitely by filling the bottles with 80 per cent. alcohol. When ready to stain, the alcohol is poured off, the sections washed with water if necessary, and then the staining solution poured into the bottle. Other reagents are used in the same manner, or sections can be treated with the reagents in flat plates, as they do not readily slip off the papers.

4. Weigert's method for a series of celloidin sections was designed especially for the nervous system and is rather complicated. The process depends on transferring the sections as cut to narrow strips of tissue-paper. To do this each section as cut is arranged in proper position close to the edge of the knife. Then a strip of tissue-paper twice as wide as the section is gently placed upon it, and the sections withdrawn from the knife. The success of the process depends on having but little alcohol on the knife, otherwise the specimen will not stick. Each specimen is placed on the paper to the right of the last one. The strips of paper when full are kept moist by being placed with the specimens uppermost on a moist surface composed of a layer of blotting-paper wet with alcohol, covered with a sheet of tissue-paper, and lying in a shallow dish.

5

When all the sections have been cut, each strip of them is taken in turn and coated on both sides with a thin film of celloidin in the following way: A strip of sections with the specimens below is first pressed gently down upon the surface of a slide covered with a thin layer of celloidin. This fastens the sections and the paper can be removed. Then a thin coat of celloidin is poured over the sections and the slide is placed on its edge to drain. When the surface of the celloidin is dry, the strips can be marked by a fine brush dipped in methylene-blue. As soon as the slides are placed in the staining solution the celloidin peels off, taking the specimens with it. Later, the strips of specimens can be divided as desired. On account of their thickness they should be cleared, after dehydrating in 95 per cent. alcohol, in a mixture of xylol 3, carbolic-acid crystals 1.

5. F. H. Verhoeff recommends this method:

In cutting the sections, the knife is not carried entirely through the celloidin block, but an uncut edge, about 3 mm. wide, is left each time. After twenty or more sections are cut in this way the knife is carried all the way through, thus producing a little book of sections. It is probably most convenient to keep each book in a separate bottle; but no difficulty is usually experienced in determining the proper order after the sections are mounted. Another way to keep them in order is to string them on a silk thread through their uncut margins. In beginning a new book a wider margin should be left for the first one or two sections, as otherwise the sections may not adhere, or the first section may be cut at double thickness. Each book is stained in the same manner as a single section, except that it is best to use slow-acting stains, so that the staining will be uniform throughout. The individual sections are not separated until the book is in alcohol preliminary to clearing. Then each section is either torn off with forceps, or the book is taken up on cigarette paper and the uncut margin removed with scissors. Each section in order is then removed, cleared quickly in oil of origanum, and placed on a slide.

6. Suzuki recommends spreading the sections out on a slide or glass plate, blotting the celloidin at one corner of the section,

and marking the number of the section on it with a certain Japanese or Chinese ink by means of a fine-pointed brush. It is said that the solid India ink freshly rubbed up with a little water is satisfactory for the purpose. The sections are placed in 80 per cent. alcohol after marking.

By the Paraffin Method.—To obtain serial sections by the paraffin method it is only necessary to avoid losing any of the sections from the ribbon as ordinarily cut. Perhaps the easiest and safest way is to cut long ribbons, a yard or more in length, and to place them on sheets of paper in proper order. They can then readily be divided by means of needles into short series of any desired number of sections, and fastened to numbered slides by means of albumin fixative.

STAINING SOLUTIONS

Hematoxylin and Hematein Stains.—The active coloring agent in most hematoxylin stains is hematein, which is gradually formed in the ordinary solutions from hematoxylin by oxidation, a process occupying a number of days or weeks and spoken of as "ripening." The selective staining power of alum-hematoxylin solutions is due to the combination of this hematein with aluminium. The resulting blue-colored solution is precipitated in the tissues (chiefly in the nuclei) by certain organic and inorganic salts there present, as, for instance, phosphates.

Mayer and Unna have shown that it is possible to oxidize and to ripen in an instant a solution of alum and hematoxylin by adding to it a little peroxid of hydrogen neutralized by a crystal of soda.

By employing hematein or its ammonium salt, instead of hematoxylin, Mayer has been able to obtain immediately ripened solutions which compare fairly favorably with old and well-known solutions prepared from hematoxylin by the slow process of ripening. They do not stain any better, however, and it is doubtful if, for the present at least, they become generally accepted.

Most solutions of alum and hematoxylin are not stable. A continuous chemical change is the formation from hematoxylin, by oxidation, of hematein, which, uniting with the alum, gives a bluish or purplish solution. The degree of blueness depends largely on the freshness of the alum. As the solution becomes older free sulphuric acid is gradually formed from the alum, causing the solution to lose its bluish or purplish tint and to become reddish. A third chemical change is the continuous formation of a precipitate due to the further oxidation of the hematein, in consequence of which it is always necessary to filter alum-hematoxylin solutions just before they are used.

More alum than is needed to combine chemically with the hematoxylin is always added to the solution, for the reason that it acts as a differential decolorizer, limiting the stain largely to the nuclei of the cells. As alum-hematoxylin solutions become older they stain more quickly, but also more diffusely. This diffuseness of staining can be counteracted by adding enough alum-water to make the stain precise again. A good alum-hematoxylin solution ought not to stain the celloidin in which the section is embedded. If the celloidin stains more or less deeply, it shows that the solution requires more alum.

Aqueous Alum-hematoxylin Solution.—

Hematoxylin crystals,	1 gram;
Saturated aqueous solution of ammonia alum,	100 c.c.;
Water,	300 "
Thymol,	a crystal.

The hematoxylin crystals are dissolved in a little water by the aid of heat. The combined solution is exposed to the light in a bottle lightly stoppered with a plug of cotton. The solution will be ripened sufficiently for use in about ten days, after which time it should be kept in a tightly stoppered bottle. The solution is very easily prepared, gives beautiful results, and will keep at its best for two to three months. The proportions of alum and of hematoxylin are the same as in Delafield's

solution. For Zenker preparations, which stain very slowly, it will be found more convenient to omit the 300 c.c. of water in the preceding formula.

Mallory's Instantaneous Alum Hematoxylin.—

Hematoxylin,	1 gram;
Ammonia alum,	10 grams;
Water,	400 c.c.;
0.25 per cent. aqueous solution of permanganate of potassium,	10 "
Thymol,	a crystal.

Pulverize the hematoxylin in a mortar and dissolve it and the alum in the water with the aid of heat. After the solution is cool add the permanganate of potassium and then the thymol. The stain is ready to use at once. If ammonium hematein is used instead of hematoxylin, take but 5 c.c. of the permanganate of potassium solution.

If a saturated solution of alum is kept on hand it may be more convenient to employ 100 c.c. of it and 300 c.c. of water in making up the staining fluid.

As the solution ripens with age and tends to stain diffusely add a little saturated alum solution to render its action more precise.

Delafield's Hematoxylin.—

Hematoxylin crystals,	4 grams;
Alcohol, 95 per cent.,	25 c.c.;
Saturated aqueous solution of ammonia alum,	400 "

Add the hematoxylin dissolved in the alcohol to the alum solution, and expose the mixture in an unstoppered bottle to the light and air for three to four days.

Filter, and add—

Glycerin,	100 c.c.;
Alcohol, 95 per cent.,	100 "

Allow the solution to stand in the light until the color is sufficiently dark, then filter and keep in a tightly-stoppered bottle.

The solution keeps well and is extremely powerful. So long as it is good the solution has a purplish tinge.

It would seem advisable, both in this solution and in Ehrlich's, to combine the alum, hematoxylin, and water, and to ripen the solution for two or three weeks before adding the other ingredients which have a tendency to prevent oxidation. A fully ripened solution would then be obtained more quickly and surely.

Harris's Hematoxylin.—

Hematoxylin,	1 gram;
Alcohol,	10 c.c.;
(Dissolve the hematoxylin in the alcohol.)	
Alum (ammonium or potassium),	20 grams;
Distilled water,	200 c.c.

Dissolve the alum in the water by the aid of heat, and add the hematoxylin solution. Bring the mixture to a boil as rapidly as possible, and then add a half gram of mercuric oxide. The solution at once assumes a dark purple color. As soon as this occurs, remove the vessel containing the solution from the flame, and cool by plunging at once into a basin of cold water. As soon as cool, the solution is ready for staining. This solution keeps for years in a well-stoppered bottle (Harris).

The addition of 4 per cent. of glacial acetic acid increases the precision of the nuclear staining.

This stain is especially adapted for sections fixed in Zenker's fluid.

Mayer's Hemalum.—

Hematein, or its ammonia salt,	1 gram;
90 per cent. alcohol,	50 c.c.;
Alum,	50 grams;
Water,	1000 c.c.;
Thymol,	a crystal.

Dissolve the hematein or its ammonia salt in the alcohol by the aid of heat, and add it to the alum dissolved in the water. The solution can be diluted with 20 parts of water or of weak alum solution.

Mayer's Acid Hemalum is prepared by adding 2 per cent. of glacial acetic acid to the above solution. The acid stain is more precise than the alkaline.

Mayer's Glycerin-alum-hematein Solution.—According to Mayer's latest investigations, glycerin is the only reliable preservative of hematein solutions. Unfortunately, it slows the staining power to a considerable extent and makes the stain less precise. He recommends the following solution for its keeping properties:

Hematein,	0.4 gram
(dissolve by rubbing up in a few drops of glycerin);	
Alum,	5 grams;
Glycerin,	30 c.c.;
Water,	70 "

Weigert's Alcohol Hematoxylin.—

Hematoxylin crystals,	10 grams;
Alcohol (absolute or 95 per cent.),	90 c.c.

The solution ripens in a week or two to a brown color, and keeps perfectly for a long time. It is used only in the Weigert stain for myelin sheaths, for which purpose it is diluted at the time of using with water and combined with carbonate of lithium (see page 143).

Mallory's Phosphomolybdic Acid Hematoxylin.—

Hematoxylin crystals,	1.75	grams;
Phosphomolybdic acid crystals,	1	gram;
Water,	200	c.c.

The hematoxylin will dissolve almost immediately if powdered, or it may be dissolved in water by the aid of heat. The solution must be exposed to the light in a bottle plugged with cotton for five to six weeks before it is fully ripened. It will keep for several months, and can be used over and over. It is employed for staining the nervous system and connective tissue.

This stain was useful after fixation in Müller's fluid. It does not give very satisfactory results after formaldehyd followed by Weigert's quick mordants.

Mallory's Phosphotungstic Acid Hematoxylin.—

Hematein ammonium,	0.1	gram;
Water,	100	c.c.;
Phosphotungstic acid crystals (Merck),	2	grams.

Dissolve the hematein in a little water by the aid of heat, and add it after it is cool to the rest of the solution; no preservative is required. If the solution stains weakly at first, it may be ripened by the addition of 5 c.c. of a $\frac{1}{4}$ per cent. aqueous solution of permanganate of potassium, or it may be allowed to stand for a few weeks until it ripens spontaneously.

Hematoxylin may be used instead of hematein ammonium, but requires 10 c.c. of the permanganate solution to ripen it.

This staining solution will be found particularly useful for the demonstration of fibrin and of neuroglia, fibroglia, and myoglia fibrils. It also brings out with great sharpness and faithfulness of detail the structures in mitotic figures, including the spindles and centrosomes.

Carmine Stains.—The active staining principle in carmine solutions is carminic acid. In cochineal carminic acid is combined with an alkaline base. Carmine itself is a commercial compound containing carminic acid combined with aluminium and calcium. Carminic acid itself does not stain, but it forms compounds with certain metals, mainly with the aluminium contained in alum, which have selective staining properties.

All of the alkaline and acid solutions made with carmine owe their staining properties to carminic acid combined with the aluminium, and perhaps also the calcium contained in the carmine.

Alum Carmine.—

Carmine,	2 grams;
Alum,	5 "
Water,	100 c.c.

Boil twenty minutes, adding enough water to make up for that lost by evaporation. When cool, filter and add a crystal of thymol to prevent the growth of mould.

Alum Cochineal.—

Powdered cochineal,	6 grams;
Ammonia alum,	6 "
Water,	100 c.c.

Boil for half an hour; add water to make up for that lost by evaporation. Filter and add a crystal of thymol.

Mayer's Alcoholic Carmine (Paracarmine).—

Carminic acid,	1.0 gram;
Chlorid of aluminium,	0.5 "
Chlorid of calcium,	4.0 grams;
70 per cent. alcohol,	100.0 c.c.

Dissolve cold or warm; allow to settle, then filter. After staining, wash out in 70 per cent. alcohol to which is added 2.5 per cent. glacial acetic acid if a more purely nuclear stain is desired.

Orth's Lithium Carmine.—

Carmine,	2.5 to 5 grams;
Saturated aqueous solution of carbonate of lithium	100 c.c.;
Thymol,	a crystal.

The carmine dissolves at once in the cold solution. When used as a counter-stain for bacteria in the Gram-Weigert method this solution should be carefully filtered, because organisms occasionally grow in it and may give rise to confusion in the stained preparations.

Neutral Carmine.—Dissolve, without heating, 1 gram of best carmine in 50 c.c. of distilled water plus 5 c.c. of strong aqua ammoniæ. Expose the fluid in an open dish until it no longer smells ammoniacal (about three days); then filter and put away in a bottle for future use. The odor of the solution will soon become bad, but the staining properties will remain unaffected.

ANILINE DYES

We have been dependent in the past on Germany for practically all our aniline dyes. Now, as the result of the late war, we are manufacturing our own. Some of them are of the highest quality, others are not so good. They are being tested by a "Commission on the Standardization of Biological Stains," an offshoot from the National Research Council, and those which meet certain specified requirements are certified. The work of certification has only just started and the results as obtained are being published in Science where they may be followed.

The important point is for the consumer to insist on certified dyes, so far as they are obtainable, when buying from the retail dealer because many poor brands of dyes, imported or manufactured by incompetents during the war, are still in the market.

Because aniline dyes are not C. P. chemicals and often contain adulterants, especially dextrin, Conn strongly advises the use of definite quantities of saturated alcoholic or aqueous solutions in all formulæ when possible, instead of so many grams, because in this way the same quantity of dye will be obtained each time.

Aniline dyes come in the form of a powder or as crystals, and most of them keep well in that condition. Methylene-blue for one, however, seems to be an exception. After the original package has been opened for a short while the dye is said to lose in intensity of staining power. It is well to keep on hand saturated alcoholic solutions of certain of the dyes, because they keep well in that form, and are ready for use when a saturated alcoholic solution is wanted. This is particularly true of methylene-blue, fuchsin (basic), and methyl-violet.

Aniline dyes are derived from either aniline or toluidin, or from both together. They may be regarded as salts having basic or acid properties. The basic colors stain cell-nuclei, including bacteria, for which they show a marked affinity. The acid colors stain diffusely. The basic dyes most commonly employed in pathological histology are methylene-blue, fuchsin,

methyl-violet, and safranin. Of the acid colors, eosin, picric acid, and acid fuchsin are most in use.

As a rule, every aniline dye has one or more standard solutions which are used largely to the exclusion of others, for the reason that, being required for certain purposes, they are kept in stock. As they are thus always at hand, they are used where simple solutions might be used. For instance, Löffler's methylene-blue solution is often used, because ready and convenient, when a simple aqueous solution would do as well.

In the following pages we have arranged under each dye the solutions of it most in use:

Methylene-blue.—1. Saturated solution in 95 per cent. or absolute alcohol. A stock solution to be used in making other solutions. It can be used as a stain by adding 1 part to 9 parts of water.

2. Aqueous solutions of various strengths are often used, and can be made up as needed.

3. *Löffler's Methylene-blue Solution.*—

Saturated alcoholic solution of methylene-blue, 30 c.c.;
Solution of caustic potash in water, 1:10,000, 100 "

This is one of the most useful of the aniline staining solutions, and will keep for a long time without losing much in staining power.

4. *Kühne's Methylene-blue Solution.*—

Saturated alcoholic solution of methylene-blue, 10 c.c.
5 per cent. carbolic-acid water, 90 "

This is a stronger staining solution than Löffler's, but the resulting stain does not seem so sharp and clear.

5. *Gabbet's Methylene-blue Solution.*—

Methylene-blue, 2 gms.
Sulphuric acid, 25 c.c.
Water, 75 "

It is used as a decolorizer and contrast-stain for tubercle bacilli.

6. *Unna's Alkaline Methylene-blue Solution.*—The strongly alkaline solution of methylene-blue recommended by Unna

for staining plasma-cells has been found extremely valuable as a general stain in connection with eosin, which should be used first. The solution should be diluted 1 : 10, or 1 : 5, for staining; it stains better after ripening for a week or two:

Methylene-blue,	1 gms.
Carbonate of potassium,	1 "
Water,	100 c.c.

(For method of using see page 100.)

7. *Unna's Polychrome Methylene-blue Solution.*—The poly-chrome methylene-blue solution, much used by Unna in various staining methods, is an old alkaline solution of methylene-blue, of which the one given above is the original formula, and in which, in consequence of oxidation, methyl-violet and methylene-red have formed. Months are required for the process of oxidation to take place at normal temperature. It may be greatly shortened by means of heat.

8. *Goodpasture's Acid Polychrome Methylene-blue Solution.*—

Methylene-blue	1 gm;
Potassium carbonate,	1 "
Distilled water,	400 c.c.

Dissolve the ingredients thoroughly and boil in a flask for thirty minutes. The methylene-blue will be polychromed and most of it precipitated. When the solution is cool add 3 c.c. of glacial acetic acid. Shake thoroughly until the precipitate is dissolved and then boil gently for five minutes or until the solution is concentrated to a volume of 200 c.c. Cool it in tap-water. It is ready for use immediately, may be used over and over, does not precipitate, and keeps indefinitely.

9. *Sahli's Borax Methylene-blue Solution.*—

Saturated aqueous solution of methylene-blue,	24 c.c.;
5 per cent. solution of borax,	16 "
Water,	40 "

Mix, let stand a day, and filter.

Fuchsin (basic).—1. Saturated alcoholic solution to be kept in stock.

2. *Ziehl-Neelson's Carbol-fuchsin.*—

Saturated alcoholic solution of fuchsin,	10 c.c.;
5 per cent. carbolic-acid water,	90 "

Carbolic acid water is made by shaking together 5 c.c. of melted carbolic-acid crystals and 95 c.c. of water. The solution should be filtered.

This solution is very powerful, stains quickly, keeps well, and can be employed for a variety of purposes.

3. *Verhoeff's Carbol-fuchsin Solution.*—

Carbolic acid, melted,	25 c.c.;
Absolute alcohol,	50 "
Fuchsin,	2 gms.

Combine the ingredients and place over night in an incubator to ensure complete solution; cool and filter. This stock solution of carbol-fuchsin, unlike the dilute aqueous solution which slowly deteriorates, is permanent and does not even require to be filtered again.

For use in staining coverslip preparations, add two drops of this stock solution to eight drops of distilled water. When larger quantities of staining solution are required, the dilution is made in the proportion of 1 c.c. of the stock solution to 6 c.c. of distilled water.

4. *Aniline-fuchsin.*—

Saturated alcoholic solution of fuchsin,	16 c.c.;
Aniline-water,	84 "

Methyl-violet.—1. Aqueous solutions of various strengths, $\frac{1}{2}$ to 2 per cent., keep well and are used for staining nuclei, bacteria, and amyloid.

2. Methyl-violet should be used instead of gentian-violet in Ehrlich's solution. Weigert recommends two permanent stock solutions by means of which the aniline methyl-violet solution can be made up easily when wanted.

Solution 1.—Absolute alcohol,	33 c.c.
Aniline,	9 "
Methyl-violet in excess.	

Solution 2.—Saturated aqueous solution of methyl-violet.

The staining solution consists of—

Solution 1,	1 part;
Solution 2,	9 parts.

This mixture will keep at the most for fourteen days.

3. For staining neuroglia-fibers Weigert employs a saturated solution made with the aid of heat in 70–80 per cent. alcohol.

Crystal-violet.—To be used in the same way as methyl-violet. It gives excellent results in the Gram staining method.

Gentian-violet.—This dye is not a definite chemical substance, but a mixture of crystal-violet, methyl-violet, and dextrin. It is better to discard it entirely, and to use either crystal-violet or methyl-violet instead in the staining solutions given; they are cited here as originally given only because they are classical.

1. Saturated alcoholic solution to be kept in stock.

2. *Ehrlich's Aniline-gentian-violet.*—

Saturated alcoholic solution of gentian-violet,	16 c.c.;
Aniline-water,	84 "

Aniline-water (aniline oil water) is made by shaking together 5 parts of aniline with 95 parts of water, and filtering the resulting milky fluid. It should come through perfectly clear. During the first few hours after the solution is made considerable precipitation takes place, so that it is best not to use it for twenty-four hours. After about ten days it begins to lose its staining power. (See under Methyl-violet, page 77.)

Zenker recommends a solution without alcohol: Dissolve the gentian-violet directly in the aniline-water. The color is said to be less easily removed from tissues when this solution is used.

3. *Sterling's Solution of Gentian-violet.*—

Gentian-violet,	5 gms;
Alcohol,	10 c.c.;
Aniline,	2 "
Water,	88 "

This solution keeps remarkably well.

4. *Carbol-gentian Violet.*—

 Saturated alcoholic solution of gentian-violet,　10 c.c.;

 5 per cent. carbolic-acid water,　　　　　　　90 "

Safranin.—Two of the many preparations by this name have been found especially useful:

 1. Safranin O soluble in water.

 2. Safranin soluble in alcohol.

The three following solutions of safranin can be thoroughly recommended:

 1. Saturated aqueous solution of "safranin O soluble in water" (to be made with the aid of heat).

 2. A mixture of equal parts of—

  A saturated aqueous solution of "safranin O soluble in water."

  A saturated alcoholic solution of "safranin soluble in alcohol."

Babes' Aniline Safranin.—

 2 per cent. aniline water,　　　　　　　　100;

 "Safranin O soluble in water,"　　　in excess.

Saturate the solution by heating it in a flask set in hot water to 60–80° C.; filter.

 This solution is extremely powerful, stains almost instantly, and will keep about two months.

Bismarck Brown.—The most common solutions are the following:

 1. A 1 per cent. aqueous solution.

 2. A saturated aqueous solution made by boiling (3–4 per cent.).

 3. A saturated solution in 40 per cent. alcohol (2–2$\frac{1}{2}$ per cent.).

 Unlike other aniline colors, Bismarck brown will keep in glycerin mounts and can be fixed in nuclei by acid alcohol. The stain is not used so much as formerly, except as a contrast stain in Gram's method and for photographic purposes.

 Other basic stains less frequently used, and then generally in aqueous solutions, are dahlia, methyl-green and iodin-green.

Pyronin.—It is used in a 1 per cent. solution in water as a counterstain in the Gram method for which purpose it is more reliable than fuchsin or safranin because it is much less likely to overpower the Gram-positive organisms and render part or all of them negative.

The dye is used also in combination with methyl-green for staining the gonococcus and the plasma cell.

Thionin.—It is generally used in an aqueous solution but this does not keep well. We have found a $\frac{1}{2}$ per cent. solution in 20 per cent. alcohol very satisfactory because it obviates this drawback.

Diffuse Stains.—1. **Eosin** is sold in two forms—as "eosin soluble in water," and as "eosin soluble in alcohol." The first is to be preferred, because a greater degree of differentiation in stain can be obtained with it. Keep on hand a saturated aqueous solution, to which a crystal of thymol has been added, and dilute with water as needed. The strength of solution to be used varies somewhat with the tissue and the reagent in which it has been fixed, but generally lies between $\frac{1}{10}$ and $\frac{1}{2}$ per cent. when the eosin is used after a hematoxylin stain. These dilute solutions should contain 25 per cent. of alcohol, otherwise they will not keep well. When eosin is employed before an aniline dye, such as methylene-blue, a 5 per cent. or even a saturated solution should be taken. Solutions of eosin should always be filtered immediately before use.

2. **Picric Acid.**—Saturated alcoholic and aqueous solutions should be kept in stock, to be diluted as needed.

3. **Acid Fuchsin.**—Aqueous solutions of various strengths are used. It is advisable to keep on hand a 5 per cent. solution and to dilute it to the strength required. A crystal of thymol should be kept in the solution because otherwise molds readily grow in it.

Altmann's Aniline Acid Fuchsin Solution.—

Acid fuchsin,	20 gms;
Aniline water,	100 c.c.

4. **Van Gieson's Picro-Acid Fuchsin Solution.**—This valuable solution was originally made by adding to a saturated

aqueous solution of picric acid enough of a saturated aqueous solution of acid fuchsin to give to the fluid a deep garnet-red color, and for certain purposes, as in staining after Zenker's fluid, this strong solution is to be preferred. Freeborn has given more precise directions for making up the solution according to the purpose for which it is to be used.

For Connective Tissue.—(See page 119.)

    1 per cent. aqueous solution of acid fuchsin,   5 c.c.;
    Saturated aqueous solution of picric acid,   100 "

For the Nervous System.—(See page 132.)

    1 per cent. aqueous solution of acid fuchsin,   15 c.c.;
    Saturated aqueous solution of picric acid,   50 "
    Water,   50 "

Picro-nigrosin (*Martinotti*).—Dissolve picric acid and nigrosin to saturation in 70 per cent. alcohol.

Combination Stains.—**Biondi-Heidenhain Staining Solution.**—

    Saturated aqueous solution of orange G,   100;
    Saturated aqueous solution of acid fuchsin or
      rubin S,   20;
    Saturated aqueous solution of methyl-green,   50.
    (About 20 gm. rubin S., 8 gm. orange G., and 8 gm.
    methyl-green; dissolve in 100 c.c. of water.)

Make up the separate solutions and let them stand for several days with excess of coloring matter (shaking the bottles occasionally) until they are saturated. Then mix the solutions. For staining, dilute the combined solution with water 1:60 to 1:100.

The following tests are used for finding out if the proper combination has been obtained: The addition of acetic acid should make the solution redder; a drop of the solution on filter-paper should make a blue spot with green in the center and orange at the periphery. If a red zone appears outside of the orange, then too much acid fuchsin is present.

Pianese's Staining Solutions and Staining Methods.—
The following stains, devised by Pianese, are recommended
by him particularly for the study of cancer, but will be found
useful in many lines of histological investigation. The first
two were used by him for tissues hardened in corrosive subli-
mate or in Zenker's fluid; the others, only after his special
fixative (given on page 49). The methods are intended for
paraffin sections:

I. Carmine and Picro-nigrosin.—1. Stain in neutral or
lithium carmine.

2. Decolorize in acid alcohol.

3. Wash in water.

4. Absolute alcohol.

5. Aniline-gentian-violet, ten minutes.

6. Iodin solution, two to three minutes.

7. Absolute alcohol, so long as any color is discharged.

8. Saturated aqueous solution of picric acid and of nigrosin,
five minutes.

9. Decolorize in a 1 per cent. alcoholic solution of oxalic
acid.

10. Water, several minutes.

11. Absolute alcohol.

12. Xylol.

13. Xylol colophonium or balsam.

Nuclei, red; cell-protoplasm, light olive-green; connective
tissue, dark olive-green; elastic fibers, bluish; bacteria and
blastomycetes, violet.

II. Methylene-blue and Eosin in Borax Solution.—Keep
three solutions on hand:

(*a*) Saturated solution of methylene-blue in a saturated
aqueous solution of borax.

(*b*) $\frac{1}{2}$ per cent. solution of "bluish eosin" in 70 per cent.
alcohol.

(*c*) Saturated aqueous solution of borax.

For use mix together 2 parts of the filtered solution *a*, 1
of *b*, and 2 of *c*. The different steps of the staining process
are as follows:

1. Absolute alcohol.
2. Staining solution, ten to twenty minutes.
3. Decolorize in a 1 per cent. solution of acetic acid.
4. Wash in water.
5. Absolute alcohol.
6. Xylol.
7. Xylol colophonium or balsam.

Nuclei, blue; red blood-globules, cell-protoplasm, granules of eosinophiles, connective tissue, etc., rose-red.

III. a. Malachite-green, Acid Fuchsin, and Nigrosin.—

Malachite-green,	1.	gm;
Acid fuchsin,	0.4	"
Nigrosin,	0.1	"
Water,	50	c.c.;
Alcohol saturated with acetate of copper,	50	"

1. Absolute alcohol.
2. Stain in 20 drops of above solution diluted with 10 c.c. of distilled water for twenty-four hours.
3. Decolorize in a $\frac{1}{2}$ per cent. aqueous solution of oxalic acid.
4. Wash in water.
5. Absolute alcohol.
6. Xylol colophonium or balsam.

Resting nuclei, light red; protoplasm, reddish yellow. In the karyokinetic figures, nuclein green; fibrillæ of the achromatic spindle and of the mitoma, bright red; centrosome and polar bodies, red; the rest of the cell-body, a reddish-yellow color.

III. b. Malachite-green, Acid Fuchsin, and Martius Yellow.—

Malachite-green,	0.5	gm;
Acid fuchsin,	0.1	"
Martius yellow,	0.01	"
Distilled water,	150	c.c.;
Alcohol, 96 per cent.,	50	"

1. Stain in the solution without diluting, half an hour.
2. Absolute alcohol.

3. Xylol.
4. Xylol colophonium or balsam.

Nuclei of resting and dividing cells, green; cell-cytoplasm, connective tissue, etc., rose-colored; "cancer-bodies," mainly red, but in masses of them some are red and some green.

IV. Acid Fuchsin and Picro-nigrosin.—

Saturated alcoholic solution of acid fuchsin,	6 drops;	
Martinotti's picro-nigrosin,	8 "	
Distilled water,	10 c.c.	

1. 70 per cent. alcohol.
2. Stain in the solution six hours.
3. Decolorize in dilute acetic acid.
4. Absolute alcohol.
5. Xylol.
6. Xylol colophonium or balsam.

Resting nuclei, red; nuclein of karyokinetic figures, yellow; cell-protoplasm, dark olive-green; "cancer-bodies," mainly olive-gray, but some or portions of them may be ruby-red.

V. Light Green (Lichtgrün) and Hematoxylin.—

Ehrlich's acid hematoxylin,	15 c.c.
Saturated solution of Lichtgrün in 70 per cent. alcohol,	5 "
Distilled water,	15 "

1. Distilled water.
2. Stain in above mixture half an hour.
3. Wash thoroughly in several waters.
4. Alcohol.
5. Oil of Bergamot.
6. Xylol colophonium or balsam.

Nuclei, green; "cancer-bodies" take the hematoxylin stain.

VI. Acid Fuchsin and Hematoxylin.—

Ehrlich's acid hematoxylin,	15 c.c.
1 per cent. solution of acid fuchsin in 70 per cent. alcohol,	15 "
Distilled water,	15 "

Stain as in V.

Nuclei, red; cytoplasm, brick-red; "cancer-bodies" take the hematoxylin stain.

Orcein, a vegetable dye obtained from certain tinctorial lichens, is used mainly for staining elastic fibers. It is soluble in alcohol, and is employed either in a neutral or acid (HCl) alcoholic solution.

Iodine is the oldest of the histological stains, but is now but little used for that purpose, except in staining amyloid.

The tincture of iodine, a saturated solution in alcohol, is used for getting rid of the precipitate of mercury formed in tissues fixed in corrosive sublimate or in Zenker's fluid.

Lugol's solution, a solution of iodine in water containing iodid of potash, is of varying strength. Iodine in this form in much used as a test for starch, amyloid, glycogen, and corpora amylacea. In Gram's stain and its modifications iodine produces some chemical change in the coloring material employed, in consequence of which, when appropriate decolorizers are used, the stain remains fast in certain structures, while from others it is easily entirely extracted.

The strength originally employed by Gram for his staining method was—

Iodine,	1 gm.;
Iodide of potassium,	2 gms.;
Water,	300 c.c.

Weigert in his modification of this method employed a stronger solution:

Iodine,	1 gm.;
Iodide of potassium,	2 gms.;
Water,	100 c.c.

Recently he has recommended the following strength both for fibrin and for neuroglia-fibers:

Iodide of potassium,	5 gms.	} saturated with iodine.
Water,	100 c.c.	

The only difference in the action of the various solutions probably is that the strong solution acts practically instantaneously, while the weaker solutions require some little time.

CLEARING REAGENTS

The object of clearing reagents is to render certain tissue-elements more prominent than others. This result may be brought about by dilute acetic acid (2–5:100), which swells up the ground substance, so that nuclei, elastic fibers, fat, myelin, and micro-organisms are more distinct, or by alkalies, which destroy the cells and ground substance and leave only elastic fibers and bacteria but little changed. This method is used almost wholly for fresh tissues.

The same result is more commonly obtained by soaking the tissues in substances which by reason of their high index of refraction render the tissues more or less transparent. Any structure which it is desirable to study is usually previously stained and thus easily rendered prominent. This second method is most applicable to hardened tissues.

For soaking and clearing the tissues a variety of reagents of different chemical properties are used. Glycerin and acetate of potash are not so much employed as formerly, because balsam mounts are more generally preferred. Of the other reagents (ethereal oils and coal-tar products), the choice depends mainly on two factors—the kind of stain which has been employed, and the substance in which the sections have been embedded. Many of the clearing reagents either dissolve celloidin or will not clear it from 95 per cent. alcohol, and nearly all of them will extract aniline colors more or less rapidly.

Most of the clearing reagents can be used after hematoxylin and carmine stains. For celloidin sections stained by either of them oleum origani cretici, oil of bergamot, or the mixture of the oils of cloves and thyme is recommended in the order given.

For aniline stains the best clearing reagent is xylol, which, however, clears directly only from absolute alcohol. It can be used, however, for celloidin or other sections dehydrated in 95 per cent. alcohol by a simple method original with Welch, and later brought into notice by Weigert. Blot the section on the slide with smooth soft filter-paper, and then pour on a

few drops of xylol; repeat the blotting, followed by xylol, two or three times, and the section will be found to be perfectly clear.

Oleum Origani Cretici.—Colorless to light brown in color; clears readily from 95 per cent. alcohol without dissolving celloidin; affects aniline colors slowly. Ordinary origanum oil is impure oil of thyme, and should not be used.

Oil of Bergamot.—Light green in color; clears quickly from 95 per cent. alcohol; does not dissolve celloidin, but after repeated use of the same lot of oil it will sometimes soften it a little. Affects aniline colors slowly, with the exception of eosin, which it extracts very quickly.

Oil of Cloves.—Straw-colored; clears quickly from 95 per cent. alcohol; dissolves celloidin; extracts aniline colors, especially methylene-blue.

Oil of Thyme.—Colorless; clears readily from 95 per cent. alcohol; makes sections brittle; does not dissolve celloidin; affects aniline colors.

Oil of Lavender.—Clears celloidin sections readily from 95 per cent. alcohol.

Oil of Cedar-wood.—Pale straw-color; clears from 95 per cent. alcohol, but, unfortunately, clears celloidin sections very slowly; does not affect aniline colors.

Aniline (*Aniline Oil*).—Colorless when perfectly pure and fresh, but soon oxidizes and turns brown; does not dissolve celloidin; clears readily from 70 per cent. alcohol; will clear from water by Weigert's method; extracts aniline colors slowly.

Xylol.—Colorless; does not dissolve celloidin; does not affect aniline colors; clears directly only from absolute alcohol; but will clear even celloidin sections from 95 per cent. alcohol if they are blotted on the slide, and the xylol is then poured over them; the process of blotting followed by xylol must be repeated two or three times.

Dunham's Mixture of the Oils of Cloves and Thyme.—Excellent for sections stained in hematoxylin or carmine. Not nearly so expensive as pure origanum or bergamot oil.

Oil of cloves,	1 part;
Oil of thyme,	4 parts.

Filter if cloudy; clears celloidin sections readily from 95 per cent. alcohol without dissolving the celloidin.

Weigert's Mixture of Carbolic Acid and Xylol.—

Carbolic-acid crystals,	1 part;
Xylol,	3 parts.

Recommended for clearing thick sections of the central nervous system after carmine and hematoxylin stains only. The next mixture is more used now-a-days.

Weigert's Mixture of Aniline and Xylol.—

Aniline,	2 parts;
Xylol,	1 part.

MOUNTING REAGENTS

The reagents most generally used for permanent mounts are Canada balsam and colophonium. Canada balsam is much the more expensive and the more difficult to prepare unless the high-priced solid form is bought. Colophonium is much the cheaper and after years of experience with it can be highly recommended. Canada balsam has the higher index of refraction, but the difference between them is slight and of no practical importance. Damar cannot be recommended because of its marked tendency to crystallize in consequence of which specimens mounted in it are often ruined.

Canada balsam occurs in commerce as a very thick, tenacious, pale, straw-colored fluid. It should be evaporated over a sand-bath to drive off all volatile substances, which might affect aniline colors, until it becomes solid and brittle on cooling. The time required is about twenty-four hours. Dissolve it then in xylol, which does not affect aniline colors, to a rather thick syrupy consistency. In this condition it is often called *xylol balsam.*

Canada balsam has such a high index of refraction that tissues mounted in it become very transparent, and only those parts are visible which are stained. Other solvents of Canada balsam, such as chloroform and benzine, may be used, but

cannot be recommended for sections stained with aniline colors. For tissue stained with osmic acid, chloroform balsam, prepared in the same way as xylol balsam, should always be used, otherwise the osmic acid stain will fade rapidly.

Canada balsam is commonly slightly acid in reaction so that it is advisable to neutralize it. The usual directions are to do this by the addition of anhydrous carbonate of potassium or sodium. It can readily be shown, however, by means of the neutral red and phenol red (phenolsulphonephthalein) indicators that these alkalies are soluble to excess in xylol and in xylol balsam and will in consequence render them alkaline. It seems advisable, therefore, to use another alkali sometimes recommended, namely, thoroughly dried, crushed but not powdered marble or calcium carbonate which will neutralize the balsam but will not make it alkaline. Shake the mixture occasionally during several days and then decant after the lime has settled out. The resulting solution is called *neutral balsam*.

Colophonium occurs commercially in the solid form: the lightest colored masses should be chosen. Two solutions should be prepared, one in xylol for aniline dyes and other stains, and one in chloroform for osmium preparations. For Wright's blood-stain use a solution of colophonium in oil of turpentine (of the best quality).

Colophonium is naturally acid and cannot be neutralized without destroying it because it consists chiefly (80–90 per cent.) of abietic acid or its anhydride and also of pinic and sylvic acids.

Oil of Cedar is recommended as the best mounting reagent after Giemsa stains. The thick evaporated form prepared for use with oil-immersion lenses should be employed.

Kaiser's Glycerin Jelly.—

Finest French gelatin,	40 gms.;
Water,	210 c.c.;
Glycerin,	250 "
Carbolic acid crystals,	5 gms.

Soak the gelatin in the water for two hours. Add the glycerin and the carbolic acid and warm for ten to fifteen minutes, stirring all the while until the mixture is smooth. It is advised to filter through the finest spun glass laid wet in a funnel. The solution will, however, filter through filter-paper in the course of twenty-four hours if placed in the paraffin oven (temperature of about 54° C.). Glycerin jelly is much to be preferred to glycerin because the mounts are practically permanent; the cover-slips are fixed. This mounting medium is very useful for the preservation of Scarlet red stains for fat in frozen sections of formaldehyde-fixed tissues.

STAINING METHODS

The purpose of staining is to render prominent the different tissue-elements, so that they may be readily recognized and studied. The constant tendency now-a-days is toward selective or differential staining methods, by which but one tissue-element will be colored to the exclusion of all others, or at least of any element that might be confused with it morphologically. These selective stains, which really are microchemical color reactions, enable us to differentiate from each other with ease and accuracy cellular and intercellular elements, or pathological products which otherwise look alike.

The list given on page 91 does not pretend to be either complete or perfect in arrangement, but will give some idea of the various elements which we wish to stain. Those for which we now possess more or less perfect differential stains are printed in *italics*.

The simplest selective stain is, of course, that for nuclei, and it can be obtained with a great variety of staining reagents. The most difficult element to stain differentially, although it can be done under certain conditions with a fair amount of success, is probably the axis-cylinder and its terminal processes.

Tissue-elements and pathological products differ from each other, not only in form and consistence, but also in chemical properties. While perfect preservation of form is sufficient

Cell.
- Nucleus.
  - Nucleolus.
  - Resting nucleus.
  - Linin.
  - Bacteria.
    - 1. Do not stain by Gram.
    - 2. *Stain by Gram.*
    - 3. *Stain by tubercle-bacillus method.*
  - *Nucleus of Ameba coli.*
  - *Centrosome and polar bodies.*
- Cytoplasm.
  - Granules.
    - *Mast cell.*
    - *Plasma-cell of Unna.*
    - *Parietal cells*, stomach.
    - *A and B islet cells*, pancreas.
    - *Zymogen granules*, intestine, pancreas.
    - Leucocytes.
      - *Five kinds of granules* described by Ehrlich.
    - *Nissl's granules in ganglion-cells.*
- Cuticle.
  - *Dendritic processes of ganglion-cells.*
  - *Axis-cylinder and terminal processes.*
  - *Contractile elements of striated muscle-cell.*
  - *Myoglia fibrils.*
  - *Red blood-corpuscles.*
  - *Blood-platelets and megakaryocytes.*
  - *Cilia of bacteria.*
  - *Certain dots or lines in ependymal cells.*
  - *So-called cilia in certain renal cells.*
  - *Bile-capillaries.*

Intercellular substances.
- *Cement substance of epithelial and endothelial cells.*
- *Ground substance of connective tissue.*
- *Connective-tissue (collagen) fibrils, and reticulum.*
- *Fibroglia fibrils.*
- *Epithelial fibrils.*
- Mucous connective tissue; *mucin.*
- *Elastic fibers.*
- Intercellular substances of cartilage.
- Ground substance of bone.
- *Myelin.*
- *Neuroglia-fibrils.*
- *Clubs of actinomycetes.*
- *Capsules of bacteria.*

Pathological products
- *Calcium*
- Hyaline substances
  - *Fibrin.*
  - *Mucin.*
  - *Amyloid.*
  - *Glycogen.*
  - *Hyalin.*
  - Colloid.
  - *Keratohyalin.*
  - *Eleadin.*
- *Fat.*
- *Hemoglobin.*
- *Hemofuscin.*
- *Hemosiderin.*
- Hematoidin.

to distinguish certain cells or elements from each other—as, for instance, polymorphonuclear leucocytes from lymphoid cells—differentiation based on micro-chemical tests is always to be preferred when possible. A few of the tests employed are colorless, like the precipitation of mucin by acetic acid. Certain tests, like the methylene-blue stain for axis-cylinders, can be applied to fresh tissues only.

Others, like the various amyloid reactions, can be obtained with fresh or hardened tissues. Most of the micro-chemical reactions, however, can be employed only with tissues which have been properly preserved. It is exceedingly important, therefore, that a tissue-element be so fixed and hardened that its peculiar chemical properties be preserved intact, otherwise a differential stain for it is impossible. Each tissue-element is a law unto itself. For example, the peculiar chemical properties of red blood-corpuscles depend on the presence in them of hemoglobin. As a differential stain of the red blood-corpuscles depends on fixing this substance in them, it is necessary to find out the chemical properties of hemoglobin, such as the fact that it is soluble in water or dilute alcohol, but not in salt solution, and that it is fixed in the red blood-corpuscles by heat, absolute alcohol and ether equal parts, corrosive sublimate, formaldehyde, bichromate of potassium, etc.

While differential stains depend in part on the chemical properties of the tissue-elements, they also depend to a certain extent on the chemical properties of the staining reagents and the decolorizers used.

Some of the tissue-elements can be stained differentially in a number of ways, sometimes after one fixing agent, sometimes after another. The simplest differential stains are those where certain tissue-elements stain directly in a given solution after they have been properly fixed. Good examples are—Ehrlich's triple stain for certain cytoplasmic granules in leucocytes, and the direct stain for elastic fibers with an acid alcoholic solution of orcein.

Other differential stains depend on the property of certain elements to hold colors they have once taken up when treated

with decolorizers. The best example of this is the tubercle bacillus, which holds certain stains through various acids or aniline hydrochlorate, followed by alcohol, and, if necessary, through a contrast-stain.

Still another varied group of elements (certain bacteria, fibrin, neuroglia-fibers, etc.) depend for a differential stain in part on changes produced in methyl-violet by iodine, in part on the decolorizer employed for extracting the coloring reagent.

Although the steps of the various staining methods differ considerably, they may be roughly arranged in the following order:

1. Staining.
2. Differentiating.
3. Decolorizing.
4. Dehydrating.
5. Clearing.
6. Mounting.

Very often two or more of the steps are combined in one, as when aniline oil is used for decolorizing, dehydrating, and clearing sections stained for certain bacteria. Sometimes the staining process occupies more than one step, as in Weigert's myelin-sheath stain. In alum-hematoxylin the differentiating reagent, the excess of alum, is combined with the stain; in Gram's method the differentiating reagent, iodine, forms a step by itself.

NUCLEAR STAINS

For general histological work no stain is so useful or can be so highly recommended as the eosin-methylene-blue stain after fixation in Zenker's fluid. It brings out nuclei and nuclear figures with great sharpness, while at the same time it stains the cytoplasm of certain cells so that they are easily distinguished from other cells. Next in point of general usefulness is phosphotungstic acid hematoxylin, owing to the sharpness with which it stains nuclei and centrosomes, and especially

nuclear figures, including the spindle. At the same time it demonstrates certain fibrils which other methods fail to show.

For class-room work alum hematoxylin, with eosin as a contrast stain, holds it own as the best general stain for celloidin sections after a variety of fixatives, but especially after Zenker's fluid.

Of the carmine stains, lithium carmine, followed by picric acid, will be found the most brilliant, generally useful, and permanent, but is useless after Zenker fixation.

Safranin gives, perhaps, the most permanent stain of any of the basic aniline dyes, and confines itself very sharply to the nuclei. It is much used after certain fixing reagents, such as Flemming's and Hermann's solutions. The Heidenhain-Biondi triple stain is useful after fixation in corrosive sublimate, but cannot be employed with celloidin sections, so that its field is limited. The other aniline dyes are used on occasion or for some definite purpose, but not so generally as those mentioned above.

A good alum-hematoxylin solution should have a bluish or purplish color, and should stain celloidin very faintly or not at all.

Aqueous Alum=hematoxylin; Mallory's Instantaneous Alum=hematoxylin; Delafield's Alum=hematoxylin; Harris's Alum=hematoxylin (see pages 68–71).

1. Stain in one of the above solutions two, five, or thirty minutes, or sometimes even longer. Sections of Zenker fixed tissue usually require at least one hour.

2. Wash in several changes of water, and then leave sections, if possible, for several hours or over night in a large dish of water; or better still, wash in running tap water for ten to thirty minutes.

3. Contrast-stain, usually an aqueous solution of eosin, $\frac{1}{10}$ to $\frac{1}{2}$ per cent., for one to five minutes.

4. Alcohol, 95 per cent., two or three changes to dehydrate and to remove excess of contrast-stain.

5. Clear in oleum origani cretici or in Dunham's oils-of-cloves-and-thyme mixture.

6. Xylol colophonium or balsam.

The more customary method of using Delafield's alum-hematoxylin solution is to filter a few drops of it into a dish of water and to stain sections for a long time, even over night, with the very dilute solution thus obtained. It is sometimes advisable to use the aqueous solution in the same way.

Mayer's Hemalum (see page 70).—1. Stain three to five minutes or longer.

2. Wash out in 1 per cent. alum solution until the stain is precise.

3. Wash thoroughly in several changes of water.

4. Alcohol, 95 per cent.

5. Oleum origani cretici.

6. Xylol colophonium or balsam.

The staining is rather diffuse, so that it has to be washed out to some extent with alum-water. Mayer's acid hemalum is more precise, and usually does not need to be decolorized, so that the second step can be omitted.

Hemalum is used for staining tissues in bulk. Twenty-four hours are required for large pieces.

Heidenhain's Hematoxylin Stain.—1. Stain twenty-four to forty-eight hours in a simple $\frac{1}{2}$ per cent. aqueous solution of hematoxylin dissolved by the aid of heat.

2. Transfer the sections directly to a $\frac{1}{3}$ per cent. aqueous solution of simple chromate of potassium for twenty-four to forty-eight hours, changing the solution frequently until no more color is given off by the sections.

3. Wash thoroughly in water.

4. Alcohol.

5. Oil.

6. Xylol colophonium or balsam.

Weigert's Iron Hematoxylin.—

Prepare two solutions:

A. Hematoxylin,   1 gram
     Alcohol, 96 per cent.,   100 c.c.
B. Liquor feri sesquichlorati,   4 "
     Water,   95 "
     Hydrochloric acid,   1 "

For use mix equal parts of *A* and *B*. The mixture is deep black and is best prepared fresh each time, although it will keep and can be used for several days.

1. Stain sections for several minutes or longer.

2. Wash in water.

3. If a counterstain is wanted, place sections for a few seconds in the following solution:

Picric acid, saturated aqueous solution,   100 c.c.
Acid fuchsin, 1 per cent. aqueous solution,   10 " "

4. Wash in water, alcohol, carboxylol, or other clearing reagent, xylol colophonium or balsam.

Heidenhain's Iron Hematoxylin.—This staining method is particularly useful for the demonstration of the centrosome, but also stains nuclei and a variety of other structures, according to the degree of differentiation.

1. Fix in corrosive sublimate, Zenker's fluid, or alcohol.

2. Stain very thin paraffin sections (not over 5 to 6μ thick) in a 2.5 per cent. solution of the violet iron alum (sulphate of iron and ammonium) for three to twelve hours. The sections should be placed vertical in the solution, so that no precipitate may fall on them.

3. Wash off quickly in water.

4. Stain in a 0.5 per cent. ripened alcoholic solution of hematoxylin for twelve to thirty-six hours.

5. Wash off in water.

6. Differentiate in the iron-alum solution, controlling the results under the microscope. The section should be washed off before each examination in a large dish of tap water, which immediately stops the decolorization.

7. Wash in running water for a quarter of an hour.

8. Alcohol, xylol, xylol colophonium or balsam.

A counterstain with Bordeaux R. before, or with rubin S. after, the iron stain is sometimes useful.

Mallory's Chlorid of Iron Hematoxylin.[1]—The results which can be obtained by this method are equally quick and

[1] Mallory: *The Journal of Experimental Medicine*, 1900, v, 18.

satisfactory after all of the usual fixing reagents except, perhaps, formaldehyde.

Celloidin or paraffin can be employed for embedding.

1. Stain sections on the slide for three to five minutes in a 10 per cent. aqueous solution of ferric chlorid.

2. Drain and blot the sections; then pour over them a few drops of a freshly prepared 1 per cent. aqueous solution of hematoxylin. If all of the hematoxylin is precipitated by the excess of ferric chlorid, pour off the solution and add a fresh supply. In three to five minutes the sections will be colored a dark bluish-black.

3. Wash in water.

4. Decolorize and differentiate in a $\frac{1}{4}$ per cent. aqueous solution of ferric chlorid. The sections should be kept constantly moving in the solution. The differentiation will be complete in a few seconds to one or more minutes.

5. Wash in water.

6. Dehydrate in alcohol.

7. Clear in oleum origani cretici.

8. Xylol colophonium or balsam.

In the above directions definite strengths have been assigned to the solutions, but they may vary greatly without affecting the result. The important point is to get the sections stained deeply, and then to decolorize slowly. The differentiation can be stopped at any moment by transferring the sections to water. Sometimes it is advisable to examine the sections under the microscope to see if enough color has been extracted.

The strength of the hematoxylin solution is unimportant; it is simply necessary to have enough hematoxylin to combine with all of the iron in and on the section. The simplest way is to dissolve by the aid of heat a pinch of the crystals in a few cubic centimeters of water. A little experience will determine about how much is needed. If a solution of hematoxylin more than one or two days old is used, the color obtained is grayish-blue, and not so bright.

This method gives a sharp, permanent, dark-blue stain to nuclei; it also stains fibrin of a grayish to dark-blue color;

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if the decolorization is not carried too far, the contractile ele-
ments of striated muscle are brought out very sharply. In
Zenker preparations the red blood-corpuscles appear of a
greenish-gray color. Connective tissue is tinted a pale yellow.
The nucleus of the amœba coli stains sharply by this method.

Carmine Stains.—The ordinary carmine solutions give
good nuclear stains, but of the finer details in a specimen they
bring out much less than a direct alum-hematoxylin stain.
They are much less used now than formerly, except as contrast-
stains to bacteria and to fibrin in the methods of Gram and
Weigert, for which purpose lithium carmine will usually give
the best results.

Alum Carmine; Alum Cochineal (see pages 72, 73).—1.
Water.

2. Stain in either of the above solutions for five to twenty
minutes.

3. Wash thoroughly in water.

4. Alcohol, 95 per cent.

5. Oleum origani cretici.

6. Colophonium or Canada balsam.

Over-staining does not occur. The solutions cannot be
recommended for tissues which stain with difficulty. When
used for staining in bulk, twenty-four to forty-eight hours are
required.

Lithium Carmine (see page 73).—1. Water.

2. Stain two to five minutes.

3. Transfer directly to acid alcohol, one or more changes
for several minutes or more, until the sections are well
differentiated.

4. Wash in water.

5. Alcohol, 95 per cent.

6. Oleum origani cretici.

7. Xylol colophonium or balsam.

This method gives an intense and permanent bright-red
nuclear stain. Over-staining is impossible. A trace of picric
acid added to the alcohol used for dehydration affords a beauti-
ful contrast-stain.

Acid alcohol,

Hydrochloric acid,	1 c.c.;
70 per cent. alcohol,	99 "

Aniline Dyes as Nuclear Stains.—Any of the basic aniline dyes may be used as nuclear stains after the following general method:

1. Stain paraffin sections in a strong solution of the dye preferred in water or in dilute alcohol for five to thirty minutes.
2. Wash in water.
3. Differentiate in 95 per cent. alcohol.
4. Dehydrate in absolute alcohol.
5. Clear in xylol.
6. Xylol colophonium or balsam.

With celloidin sections use 95 per cent. alcohol, blot with filter paper, and clear in xylol.

As a matter of fact, however, certain dyes and certain solutions are generally used in preference to the others. Most of the colors are more or less affected by all clearing reagents except xylol. With paraffin sections and those from which the celloidin has been removed it is very easy to dehydrate in absolute alcohol and to clear in xylol. With celloidin sections, however, this is impossible, because the absolute alcohol will dissolve out the celloidin, and this is usually not desirable. For celloidin sections, therefore, blot with filter paper, and then pour on xylol; repeat the blotting, followed by xylol, two or three times until the specimen is perfectly clear. Mount in xylol balsam.

In washing out the excess of color it is sometimes found advantageous to acidulate very slightly either the water or the first alcohol with acetic or hydrochloric acid. This process, if not carried too far, tends to make the nuclear stain sharper.

Safranin is one of the very best nuclear-staining aniline dyes. Tissues may be hardened in alcohol, corrosive sublimate, Flemming's, Hermann's, or Zenker's fluids. Any one of the solutions of safranin given on page 79, may be used.

1. Stain paraffin sections two to five minutes to twenty-four hours according to the staining solution and fixing reagent used.

2. Wash in water.

3. Absolute alcohol, several changes, until the section appears properly differentiated.

4. Xylol.

5. Xylol colophonium or balsam.

For celloidin sections dehydrate in 95 per cent. alcohol, and clear by the xylol blotting-paper method. To render the stain more precise, a few drops of acid alcohol are sometimes added to the first alcohol.

Mallory's Eosin and Methylene-blue Stain.—This stain, used on paraffin sections of tissues fixed in Zenker's fluid, can be recommended as the very best general stain yet devised. It is a sharp nuclear stain, and, at the same time, brings out with a great deal of differentiation all the various other structures in the different tissues. It has been in constant use for many years as the routine stain for all tissues in the pathological laboratories of the Harvard Medical School and Boston City Hospital.

Fix in Zenker's fluid.

1. Stain paraffin sections in a 5 per cent. aqueous solution of eosin for twenty minutes or longer. Sometimes it is advisable to get a deeper eosin stain by placing the sections in the paraffin oven for thirty to sixty minutes.

2. Wash in water to get rid of excess of eosin.

3. Stain in Unna's alkaline methylene-blue solution (see page 76), diluted 1–4 or 5 with water, for thirty to sixty minutes. Pour the solution off and on the sections several times.

4. Wash in water.

5. Differentiate and dehydrate in a dish of 95 per cent. alcohol, keeping the section in constant motion, so that the decolorization shall be uniform. Control the result under the microscope. When the pink color has returned to the section and the nuclei are still a deep blue, finish the dehydration quickly with absolute alcohol.

6. Xylol.

7. Xylol colophonium or balsam.

For celloidin sections use 95 per cent. alcohol, blot, and pour on xylol; repeat the last two steps until the specimen is clear.

It is important to get a deep stain with eosin, because the methylene-blue washes it out to a considerable extent. The eosin must be used first, because methylene-blue is readily soluble in an aqueous solution of eosin, and therefore is quickly extracted if the eosin is used after it, while on the other hand eosin is very slightly soluble in an aqueous solution of methylene-blue which is precipitated by any excess of eosin.

The success of this staining method has been found by Wolbach to depend on the presence of colophonium in the alcohol used for differentiation. This is present in alcohol obtained from the barrel, but not in alcohol preserved in glass. It must, therefore, be added. This is most easily done by keeping on hand a 10 per cent. solution of colophonium in absolute alcohol, and adding a few drops of it to the alcohol in which the sections are differentiated. Wolbach has also shown that sections fixed in formaldehyde may be stained by this method, provided the amount of colophonium in the alcohol be increased to from 3 to 10 per cent.

During the past few years there have been many complaints about the eosin-methylene-blue staining method. The sections appeared muddy; the details were not sharp and brilliant. Apparently the trouble has been due to the poor quality of the eosins which have been put on the market since the war began. With eosins of best quality now available the former good stains are again easily obtainable.

As Unna's solution ages and ripens the staining power of the methylene-blue, which alone is wanted in this method, rapidly weakens. On this account it has been found advisable to keep the dye and the alkali in separate solutions and to combine them only as wanted for use.

Solution *A*.	Methylene-blue,	2 grams.
	95 per cent. alcohol,	10 c.c.
	Water,	90 "
Solution *B*.	Carbonate of potassium,	1 gram.
	Water,	1000 c.c.

Combine in the proportion of one part of the dye to nine parts of the alkali.

A single solution which does not ripen and change, at least under ordinary conditions, and which, therefore, holds its strength and gives constantly good results is the following:

Methylene-blue,	1 gram.
Borax,	1 "
Water,	100 c.c.

For staining take one part of this solution to five of water.

Diffuse or contrast=stains are useful to make prominent certain of the tissue-elements left uncolored by the nuclear stain. A greater richness of detail is obtained with diffuse stains if, after rather deep staining, the sections are washed out for some time in alcohol, because certain structures possess a greater affinity than others for certain diffuse stains, and by holding them are brought out sharply.

Of the diffuse stains, eosin, picric acid, and acid-fuchsin in Van Gieson's mixture are the ones most frequently employed.

Eosin is most frequently used as a contrast to alum-hematoxylin and methylene-blue stains, but is often serviceable with alum-cochineal, methyl-violet, etc. It brings out particularly well red blood-corpuscles and smooth and striated muscle-fibers. The strength of the solutions used after hematoxylin varies from $\frac{1}{10}$ to $\frac{1}{2}$ per cent., according to the tissue and the fixative used. Zenker preparations stain intensely in eosin, so that for them a very dilute solution is advisable. When desired as a contrast-stain to basic aniline dyes, eosin should be used first in a 5 per cent. solution, because otherwise it is likely to be washed out by the nuclear stain.

Picric acid is used for contrast with the carmine stains, more rarely with alum-hematoxylin. Striated muscle-fibers and cornified epithelium are rendered especially prominent by it. To stain with picric acid it is only necessary to add a few drops of a saturated aqueous solution to a dish of water, or of a saturated alcoholic solution to a little alcohol, and allow sections to remain in the solution for a few seconds.

Van Gieson's stain (see p. 80), a mixture of picric acid and acid fuchsin, is excellent as a contrast-stain to alum-hematoxylin, especially when it is desirable to render prominent connective-tissue fibrillæ or certain pathological products. The nuclear stain with alum-hematoxylin must be rather deep, because the picric acid to some extent extracts or overpowers it.

1. Stain deeply in alum-hematoxylin.
2. Wash in water.
3. Stain in Van Gieson's solution three to five minutes.
4. Wash in water and dehydrate directly in
5. Alcohol, 95 per cent.
6. Oleum origani cretici.
7. Xylol colophonium or balsam.

Neutral Carmine (see page 73).—Neutral carmine is a diffuse stain, and is employed more especially for the central nervous system and for bone.

Filter one or two drops of the solution into 20 c.c. of distilled water, and leave the sections in the dilute solution over night. It is advisable to place a piece of filter-paper on the bottom of the dish for the sections to rest on, otherwise they may be stained on the upper side only. In double stains with hematoxylin and carmine the sections should be stained first in the hematoxylin and then thoroughly washed in water for six to twelve hours before they are stained in the carmine. After the carmine they are again to be thoroughly washed in water.

Combination Stains.—Biondi-Heidenhain Stain (see p. 81).—Tissues must be hardened in corrosive sublimate.

1. Stain paraffin sections six to twenty-four hours with the dilute solution.
2. Wash out a little in 90 per cent. alcohol.
3. Dehydrate in absolute alcohol.
4. Xylol.
5. Xylol colophonium or balsam.

It is important to place the sections directly from the staining fluid into the alcohol, because water washes out the methyl-green almost instantly.

Staining in Mass.—The staining of tissues in mass is a procedure much less employed in pathological than in normal histology, but still occasionally useful. For pathological tissues a variety of stains is generally necessary. It is therefore much better to make a series after one of the methods described, and then to stain the sections in whatever way seems best.

For staining in bulk, only a limited number of solutions are available—either those, like alum-carmine and alum-cochineal, which do not stain beyond a certain point, or those, like lithium and borax-carmine and Heidenhain's hematoxylin, which may be decolorized so as to leave only the nuclei stained. The process of staining differs from that for sections only in the length of time required for each step. Tissues $\frac{1}{2}$ cm. thick will need from one to two days in the staining solution.

MITOSIS

For the study of karyomitosis it is important that the tissue be perfectly fresh—that is, just removed from a living animal or from one just dead—and that it be fixed in a suitable reagent as quickly as possible. The best results cannot be obtained with tissues put into a hardening fluid over half an hour after removal from a living animal. On the other hand, mitotic figures can be demonstrated in tissues which have been dead for some time (twenty-four hours or more) before being put into a fixing reagent, but the details of the figures are not so perfect as those in absolutely fresh tissues, and the figures are not so numerous, because some of them have completed their changes and can no longer be recognized. It is therefore evident that mitosis can be studied much better in tissues from the lower animals, or in tissues removed by operation from the human body, than in the organs and tissues removed at post-mortem examinations.

The choice of fixing reagents for the study of mitotic figures is important. The figures can often be demonstrated after hardening in alcohol or even in Müller's fluid, but for their careful study quicker and more perfect fixing reagents must

be used. Nearly all of the reagents employed penetrate slowly, so that it is absolutely necessary for the best results that the tissue to be hardened be cut into very thin slices, rarely over 4 mm. in thickness and preferably not over 2 mm. The amount of fixing reagent used should always be at least ten to fifteen times as great as the volume of the tissue, and should be changed if it becomes cloudy.

The most important fixing reagents are—

1. Flemming's solution.
2. Hermann's solution.
3. Pianese's solution.
4. Zenker's fluid.
5. Corrosive sublimate.
6. Orth's fluid.

The first three solutions penetrate with much difficulty, so that tissues placed in them should be especially thin. The most generally useful stain for mitosis is probably safranin. The time of staining varies with the solution used. Babes' is the quickest. The mitotic figures should be stained deeply: then, when treated with alcohol slightly acidulated with hydrochloric acid, they will retain the color, while the resting nuclei will yield up most of theirs and become very pale or even colorless. In consequence of this intense stain mitotic figures can then be very readily found.

Fixation in Zenker's fluid and staining in phosphotungstic-acid hematoxylin can be highly recommended. Centrosomes and spindles are brought out with great distinctness.

Directions for Staining Karyomitotic Figures with Safranin.—1. Stain paraffin sections five minutes to twenty-four hours, according to solution used.

2. Wash in water.

3. Wash in 95 per cent. alcohol to which are added a few drops of acid alcohol.

4. Wash in pure 95 per cent. alcohol, followed by absolute alcohol.

5. Xylol.

6. Xylol colophonium or balsam.

For celloidin sections dehydrate in 95 per cent. alcohol, blot, and pour on xylol; repeat the last two steps until the specimen is clear. Safranin can be used after any of the above fixing reagents.

Other useful stains are carbol-fuchsin and aniline-methyl-violet, used in the same way as the safranin. The Gram-Weigert method gives good results after Flemming's solution.

After fixing in corrosive sublimate mitotic figures can be demonstrated by the Biondi-Heidenhain solution, which stains resting nuclei blue-violet and mitotic figures green. After Pianese's solution his special staining mixtures should be used (see page 82). His methods are said to give beautiful results.

METALLIC STAINS OR IMPREGNATIONS

Experimental investigation has shown that certain metals can be used for staining certain tissue-elements, either because they are directly reduced from solutions of appropriate salts or because they are taken up and retained by certain tissue-elements, which are rendered prominent when the metallic salt is reduced later. The most valuable metals for this purpose are silver, gold, and osmium.

Silver is used generally in the form of silver nitrate, to stain of a brown or dark-brown color the cement substance between epithelial and endothelial cells and the ground substance of connective tissue. The method finds its chief use in pathology in demonstrating the endothelial covering of a doubtful surface, in outlining the endothelial cells of pathologically altered blood- and lymph-vessels, in demonstrating the treponema pallidum by Levaditi's method, and in staining the ground substance of the connective tissue of the cornea when that organ is used experimentally for the study of inflammation. In combination with certain other salts, especially bichromate of potassium, nitrate of silver is much employed in the Golgi methods to stain ganglion-cells and their processes in the central nervous system.

The difficulty of the silver method lies in the fact that the salt forms with albuminous fluids granular and thread-like coagula which can easily give rise to false pictures. For this reason the method is limited almost entirely to natural surfaces, which should be washed off with water or a 2 per cent. solution of nitrate of sodium before the silver solution is applied. It is generally advisable to use the nitrate of silver in a very dilute solution, 1 : 250 or 500. The solution is allowed to act on the surface for about a minute, and is then washed off with water. The tissue is next exposed in water to the action either of sunlight or of diffuse light. The outlines of the cells soon appear as dark lines, brown to black in color. The tissue to be stained should be kept stretched, because a precipitation of the silver occurs wherever there is a fold in the surface. Although nitrate of silver penetrates but a slight distance, it is possible to stain the outlines of the endothelial cells of the lymphatics and blood-vessels as well as the ground substance of the connective tissue—in a rabbit's diaphragm, for instance—by treating the upper or lower surface with the silver solution. The thoracic organs should be removed, and then the upper surface of the tendinous portion of the diaphragm left *in situ* is exposed to the action of the silver salt in the manner already described.

The outlines of the endothelial cells of blood-vessels are usually stained by injections of the silver salt through an artery. In the same way the limits of the epithelial cells of the alveoli of the lung can be stained by injections through a bronchus.

Although generally employed in solution, nitrate of silver is sometimes used in the solid form, and for the cornea this method is preferable. Chloroform the animal, preferably a rabbit, deeply; rub the cornea with a stick of nitrate of silver hard enough to remove the surface epithelium. Allow the salt to act about ten minutes, then kill the animal, remove the eye, cut out the cornea, wash it, and expose to diffuse daylight for half an hour. It is then placed in a mixture of glycerin and water, 30 parts to 70, very slightly acidulated with acetic acid

(about ₁'₀ per cent.) for twenty-four hours, so as slightly to swell and to soften the tissues. Sections of the cornea are best made with the freezing microtome. Incise the periphery a little at four points equally distant from each other, so that the cornea will lie flat. A direct stain with alum-hematoxylin gives by all odds the best results. The sections may be mounted in glycerin or balsam. The latter method is perhaps the better. Dehydrate the sections in 50 per cent., then in 70 per cent., alcohol, clear in aniline oil, wash with xylol, and embed in balsam. This method avoids the shrinkage which is caused by using strong alcohol.

Gold, in the form of the simple or double chlorid, is employed to stain the cytoplasm of cells of connective tissue, and more particularly the axis-cylinders of nerve-fibers and their terminal processes. Like nitrate of silver, it acts as a fixing and hardening reagent as well as a stain. Unfortunately, it penetrates tissues but a very slight distance, and, so far as staining is concerned, is inconstant in action. Its chief use in pathology is in connection with experimental work on the cornea and in regeneration. The conditions under which the reduction of the gold salt takes place are not exactly understood, but both penetration and reduction are aided by the action of organic acids, such as formic, citric, and tartaric acids, on the tissues both before and after the treatment with the gold salt. Of the many methods proposed, the following are recommended:

Löwit's Formic-acid Method.—1. Place very small bits of fresh tissue in a mixture of formic acid 1 part, and water 1 to 2 parts, until they become transparent (a few seconds to several minutes).

2. Transfer to chlorid of gold, 1 to 1.5 parts to 100 of water, for fifteen minutes.

3. Formic acid, 1 part to water 3 parts, for twenty-four hours.

4. Concentrated formic acid twenty-four hours. Preserve in glycerin or balsam.

All the steps except the first should be performed in the dark.

Ranvier's Formic-acid Method.—1. Boil together 8 c.c. of a 1 per cent. solution of chlorid of gold and 2 c.c. of formic acid. When the solution is cold place very small bits of tissue in it for one hour, in the dark.

2. Wash quickly in water.

3. Expose to diffuse light in a mixture of formic acid 10 c.c. and water 40 c.c. Reduction takes place slowly (twenty-four to forty-eight hours).

4. Harden in 70 per cent., then 90 per cent., alcohol in the dark.

Osmic acid (perosmic acid, osmium tetroxid) is used as a fixing reagent and for staining fat and myelin, by which it is reduced. As osmic acid is quickly reduced by organic substances, care must be taken in making up the solution. Remove the label from the sealed tube in which the acid comes, and place the tube, after cracking off one end, in a glass-stoppered bottle containing enough water to make a 2 per cent. solution. If desired, the tube can be broken after it is in the bottle by violent shaking. It should be borne in mind that osmic acid is very irritating to the bronchial mucous membrane.

In a 1 or 2 per cent. solution osmic acid is used to stain fat in teased preparations or frozen sections of fresh tissues. In Marchi's method it is used to stain fat in tissues which have been hardened for some time in Müller's fluid. As a fixing reagent it is usually combined with other reagents, as in Flemming's solution, both for its property as a fixative and for the purpose of staining any fat present.

Preparations stained in osmic acid may be kept indefinitely in alcohol. When sections are mounted they should be cleared in chloroform, and preserved in chloroform balsam prepared in the manner described elsewhere. Xylol and other clearing reagents cause the stain to fade.

SPECIAL STAINS FOR CERTAIN TISSUE=ELEMENTS OTHER THAN NUCLEI

MITOCHONDRIA

Mitochondria[1] is a term applied to certain threadlike granules, which occur in the cytoplasm of many kinds of cells and which have received various names. They may be round or oval, rod-shaped, or in the form of filaments. They disappear quickly after death and are destroyed by acetic acid and most fixing solutions. Special fixatives and staining methods are required for their demonstration. The best method is that devised by Bensley.

A. Acetic-osmic bichromate fixation.

1. Fix very thin sections of perfectly fresh tissue (1 mm. thick) for twenty-four hours in the following solution:

Osmic acid, 2 per cent. solution,   2 c.c.;
Potassium bichromate, 2.5 per cent. solution, 8 "
Glacial acetic acid,   1 drop.

2. Wash in distilled water for one hour.

3. Dehydrate in graded alcohols, 50, 70, 95, and 100 per cent., twenty-four hours each.

4. Embed in paraffin.

5. Cut sections not over 4 microns thick and attach them to the slide by the albumin-water method.

B. Staining.

1. Pass sections through toluol or xylol and graded alcohols to water.

2. One per cent. solution of permanganate of potassium for thirty to sixty seconds.

3. Five per cent. solution of oxalic acid for thirty to sixty seconds.

4. Wash thoroughly in water.

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[1] Cowdry, E. V., "The Relation of Mitochondria and Other Cytoplasmic Constituents in Spinal Ganglion Cells of the Pigeon," *Internat. Monatsschrift f. Anat. u. Phys.*, 1–32, xxix, 1912.

5. Stain for six minutes at 60° C. in Altmann's aniline-acid fuchsin solution.

Aniline water,	100 c.c.;
Acid fuchsin,	20 grams.

6. Rinse in distilled water.

7. Differentiate by dipping for an instant in a 1 per cent. aqueous solution of methyl green.

8. Drain and dehydrate directly and quickly in absolute alcohol.

9. Clear in toluol or xylol and mount in balsam. The mitochondria are stained intensely red, the nuclei green.

GOLGI'S INTERNAL APPARATUS OR THE RECTICULAR MATERIAL

Da Fano's Modification of Cajal's Silver Impregnation Method.[1] *Fixation.*—1. Fix thin slices of fresh tissue for 6–8 hours or more, but never over 24 hours, at room temperature in—

Distilled water,	100 c.c.;
Cobalt nitrate,	1 gram;
Formalin,	15 c.c.

The solution will keep unaltered for months. Neutralize the formalin if strongly acid. For very delicate tissues reduce the amount of formalin.

2. Wash quickly in distilled water.

Impregnation

3. Place in a 1.5 per cent. solution of silver nitrate for 24–48 hours at room temperature in the dark.

4. Wash quickly in distilled water.

[1] Da Fano, Proc. Physiol. Soc., Journ. Physiol., LIII, 1920; Journ. R. Micr. Soc., 1920, p. 157.

Reduction

5. Place in Cajal's reducing fluid, freshly prepared each time, for about 12 hours.

Distilled water,	100 c.c.;
Hydrochinon,	1.5–2 grams;
Anhydrous sodium sulphite, .	0.25–0.5 " "
Formalin,	6 c.c.

Embedding, Toning and Counterstaining

6. Wash in distilled water long enough to remove formalin.

7. Pass through graded alcohols.

8. Clear by the oil of cedar method.

9. Embed in paraffin.

10. Cut, attach to slide, clear in xylol and mount in xylol colophonium or balsam.

The reticular material appears black or dark brown on a yellow to buff back-ground. The stains do not keep well; hence it is better, after attaching the sections to the slide to bring them back through xylol and graded alcohols to distilled water, to tone them by immersion in a 0.1–0.2 per cent. acid solution of gold chlorid followed by fixation in a 5 per cent. solution of sodium hyposulphite, after which they can be counterstained if desired in alum carmine, dehydrated, cleared and mounted.

CYTOPLASMIC GRANULES

To study the albuminous granules in fixed tissues it is necessary to employ reagents which do not dissolve them; therefore, solutions containing acetic acid must be avoided. Tissues fixed in Zenker's fluid show the granules well preserved only at the periphery. Farther in where the acetic acid reached them before the other chemicals did the granules will be found to have disappeared more or less completely. The best means yet devised for demonstrating them seems to be the following.

The Altmann-Schridde Method for Demonstrating Granules in Cytoplasm.—1. Fix thin slices of perfectly fresh tissue,

preferably removed at operation, in Orth's fluid (formaldehyde-bichromate mixture) for twenty-four hours. The tissue can be fixed first in 10 per cent. formaline but must be transferred later to a 2.5 per cent. solution of bichromate of potassium for twenty-four hours. Distilled water must be used in making the solutions.

2. Wash thoroughly in frequent changes of distilled water for twenty-four hours.

3. Place in a 1 per cent. aqueous solution of osmic acid for twenty-four hours in the dark.

4. Wash thoroughly in running water for twenty-four hours.

5. Dehydrate in the dark in graded alcohols running from 50 per cent. up to absolute.

6. Embed in paraffin by the chloroform method.

7. Cut very thin sections (1–2 microns) and attach them to the slide by the egg-albumen method.

8. Transfer through chloroform and graded alcohols to distilled water.

9. Stain in Altmann's aniline-water acid fuchsin solution steaming for 5–15 minutes and then allow to cool.

10. Drain off stain and differentiate in the following solution:

Saturated alcoholic solution of picric acid, 1 part;
20 per cent. alcohol, 7 parts.

until the section appears of a clear yellowish red color.

11. Dehydrate in 96 per cent. and absolute alcohol, clear in chloroform and mount in chloroform colophonium or balsam.

The granules of the various cells show different shades of color: the neutrophilic granules appear brownish red, the eosinophilic dark red, the plasma cell granules brick red, the mast-cell granules dark gray, the basophilic granules unstained but recognizable.

MAST=CELLS

Mast-cells are found in the tissues under a variety of conditions, both normal and pathological. They are often numerous in chronic inflammatory processes and occasionally occur abundantly in leiomyomata. Their cytoplasmic granules stain

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intensely, like bacteria with the basic aniline dyes, especially after fixation in alcohol or formaldehyde. In tissue preserved in Zenker's fluid they usually do not stand out prominently. In eosin-methylene-blue preparations they appear like poorly stained eosinophiles. Several methods of staining the granules are given. With Unna's stains for plasma-cells a differential color-stain is obtained for the granules of the mast-cells.

Ehrlich's Method.—*A. General Stain.*—Harden in alcohol.

1. Stain with a saturated aqueous solution of dahlia.

2. Wash out with acidified water.

3. Dehydrate in 95 per cent. alcohol, absolute alcohol, xylol, xylol balsam.

B. Specific Stain.—Only the cytoplasmic granules are stained. Harden in alcohol.

1. Stain twelve hours in—

Absolute alcohol,	50 c.c.
Water,	100 "
Glacial acetic acid,	12.5 "
Dahlia,	q.s., so that the

solution is almost saturated.

2. Wash out in 95 per cent. alcohol, absolute alcohol, xylol, xylol balsam.

C. Ehrlich-Westphal Method.—Nuclei red; granules blue. Harden at least a week in alcohol.

1. Stain in the following solution twenty-four hours:

Alum-carmine solution,	200
Saturated solution of dahlia in absolute alcohol,	200
Glycerin,	100
Glacial acetic acid,	20

(Stir repeatedly, then allow the mixture to stand for some time.)

2. Decolorize for twenty-four hours in absolute alcohol.

3. Xylol, xylol balsam.

Unna's Isolated Stains for Mast-cells.—Harden in alcohol. Nuclei blue; cytoplasmic granules of mast-cells red.

A.—1. Stain in polychrome methylene-blue solution, plus a little alum, for three hours to over-night.

2. Wash in water.

3. Absolute alcohol, xylol, xylol balsam.

B.—1. Stain in polychrome methylene-blue solution one-quarter of an hour.

2. Wash in water.

3. Decolorize in glycerin-ether mixture for five to ten minutes.

4. Wash a long time in water.

5. Absolute alcohol, xylol, xylol balsam.

ENDOTHELIAL LEUKOCYTES

McJunkin's Method of Demonstrating Phagocytosis by Means of Lampblack[1]

The technique was worked out for the phagocytic mononuclear (endothelial) leukocyte of the blood but can be applied to the bone marrow.

For Diluted Blood.—*Lampblack Suspension in Sodium Citrate.* —3.8 per cent. sterile sodium citrate solution containing 1 part by weight of a good grade of lampblack. An even suspension is secured by vigorous shaking.

Withdrawal of Blood.—From punctured wound of finger tip after sterilization with tincture of iodine, followed by 95 per cent. alcohol.

Procedure.—A. 1. To sterile 15 c.c. graduated centrifuge tube add 2 c.c. of sterile sodium citrate-lampblack suspension.

2. Make up to 5 c.c. by dropping directly into it from finger 3 c.c. blood. Flow of blood accentuated by squeezing finger.

3. Shake vigorously.

4. Filter into second centrifuge tube through laundered muslin moistened with sodium citrate and depressed into it.

B. 1. Centrifuge at moderate speed for 15 minutes, at high speed for 5 minutes.

2. Suck black, leucocytic layer on surface into large bore hemocytometer pipette after moistening pipette with sodium citrate solution.

[1] McJunkin, F. A., "A Simple Technic for the Demonstration of a Phagocytic Mononuclear Cell in Peripheral Blood," Arch. Internal Medicine, 1918, xxl, 59–65.

3. Stretch wide rubber band over ends of pipette and shake for one minute.

4. Incubate in horizontal position at 37.5° C. for one hour shaking every quarter hour.

5. Remove, shake for five minutes and make cover-glass preparations.

Staining.—Stain in usual way with any polychrome blood stain.

For Undiluted Blood.—*A. Preparation of lampblack-sodium citrate powder.*

1. Grind 10 gms. of sodium citrate (Merck) U. S. P. to small granules (not powder) in mill.

2. Grind in mortar for thirty minutes 0.5 gm. of lampblack.

3. Mix intimately 7.7 gms. of sodium citrate with 0.3 gm. of lampblack.

4. Place in bottle and keep in desiccator.

B. Preparation of Test Tube.—1. From glass tubing make test tube 45 mm. long, having 7 mm. bore.

2. Graduation of tube. Put 8 mg. of the dry lampblack-sodium citrate powder, 4 small glass beads, and 1 c.c. of water into tube. Shake and mark level. Empty, wash and dry tube and beads.

Procedure.—1. Replace beads.

2. Add 8 mg. of lampblack-sodium citrate powder.

3. Obtain blood as before from finger. Let blood touch apex of V-shaped wire inverted into test tube to avoid contact with its mouth. Fill to mark.

4. Remove wire and stopper with rubber cork.

5. Shake for 5 minutes and centrifuge at moderate speed for 15 minutes.

6. Incubate at 37.5°C. for thirty minutes.

7. Remove and shake for two minutes.

8. Centrifuge as before.

9. Repeat steps 6 and 7.

10. Make cover-glass preparations.

11. Dry in air for three hours and stain in usual manner.

PLASMA=CELLS

Plasma-cells arise from lymphocytes. They are often abundant in subacute and chronic pathological processes, and are characterized by cytoplasm which stains quite deeply in alkaline methylene-blue solutions. The eosin-methylene-blue stain after fixation in Zenker's fluid brings them out very sharply. The two methods best suited for their demonstration furnish at the same time a differential color-stain for mast-cells. The granules of the latter are stained red, the plasma-cells are stained blue.

Unna's Differential Stains for Plasma-cells and Mast-cells.—Harden tissues in absolute alcohol.

A.—1. Stain paraffin sections in polychrome methylene-blue one-quarter of an hour to over-night.

2. Decolorize in a small dish of water, to which are added a few drops of glycerin-ether mixture.

3. Wash thoroughly in water.

4. Absolute alcohol, xylol, balsam.

B.—1. Stain in polychrome methylene-blue solution five to fifteen minutes.

2. Wash in water.

3. Decolorize and dehydrate in a $\frac{1}{4}$ per cent. alcoholic solution of neutral orcein (about fifteen minutes).

4. Absolute alcohol, xylol, balsam.

Methyl-green-Pyronin Stain (Unna-Pappenheim).—

Methyl-green,	0.15
Pyronin,	0.25
Alcohol,	2.50
Glycerin,	20.00
0.5 per cent. carbol-water to	100.00

Fix in alcohol, stain five to ten minutes in incubator, wash in cold water, differentiate and dehydrate quickly in absolute alcohol, clear in xylol, and mount in xylol balsam. Acetone can be used to advantage in place of alcohol to dehydrate, because it has less tendency to extract the pyronin from the cytoplasm of the cells.

THE COLLAGEN FIBRILS AND RETICULUM OF CONNECTIVE TISSUE

Several methods are available for the demonstration of collagen fibrils and reticulum. The simplest is by means of Van Gieson's picric acid and acid fuchsin solution, but it is applicable to the coarser fibers only. The stain with aniline blue is believed to be better than any yet proposed, but is limited to tissues hardened in Zenker's fluid.

A. **Mallory's Aniline Blue Stain.**—The following method is not absolutely differential because, besides collagen fibrils and reticulum, it also stains certain hyaline substances, but these latter are usually so different morphologically that confusion cannot arise. The method is also useful for the study of fibrin, fibroglia fibrils, smooth and striated muscle-fibers, and amyloid.

1. Fix in Zenker's fluid.
2. Embed in celloidin or paraffin.
3. Stain sections in a 0.5 per cent. aqueous solution of acid fuchsin for five minutes or longer, depending on the freshness of the tissue.
4. Transfer directly to the following solution and stain from ten to twenty minutes or longer:

Aniline blue soluble in water,	0.5
Orange G,	2.0
One per cent. aqueous solution of phosphomolybdic acid,	100.00

5. Wash and dehydrate in several changes of 95 per cent., then absolute alcohol.
6. Clear in xylol.
7. Xylol colophonium or balsam.

For celloidin sections use 95 per cent. alcohol and clear by the xylol blotting-paper method.

The collagen fibrils and reticulum and connective tissue, amyloid, mucus, and certain other hyaline substances stain blue; nuclei, cytoplasm, fibroglia fibrils, axis-cylinders, neuroglia-fibers, and fibrin red; red blood-corpuscles and myelin-

sheaths yellow; elastic fibers pale pink or yellow. The various structures do not stain with equal intensity, so that certain ones are brought out with great sharpness. This is particularly true of the collagen fibrils and reticulum of connective tissue, and of fibrin and smooth and striated muscle-fibers.

If it is desired to bring out the collagen fibrils as sharply as possible, omit the staining with acid fuchsin. Then the nuclei and protoplasm stain yellow, and the blue fibrillæ and reticulum stand out more prominently.

B. **Van Gieson's Stain.**—The proportions given are those recommended by Freeborn. Occasionally it will be found necessary to increase the proportion of the acid fuchsin.

1. Harden in chrome salts or in corrosive sublimate. The results after alcohol are not so good.

2. Stain deeply in alum-hematoxylin.

3. Wash in water.

4. Stain for three to five minutes in

> 1 per cent. aqueous solution of acid fuchsin,   5 c.c.;
> Saturated aqueous solution of picric acid,   100 "

5. Dehydrate in 95 per cent. alcohol.

6. Oleum origani cretici.

7. Xylol colophonium or balsam.

C. **Unna's Orcein Stain.**—1. Harden in alcohol.

2. Stain in the concentrated solution of polychrome methylene-blue five minutes.

3. Wash in water.

4. Decolorize, differentiate, and stain in a 1 per cent. solution of orcein in absolute alcohol fifteen minutes.

5. Wash in absolute alcohol.

6. Xylol.

7. Xylol colophonium or balsam.

Nuclei, dark blue; cytoplasm, pale blue; elastic and connective-tissue fibers, deep orcein red; smooth muscle-fibers, bluish; mast-cell granules, red; cytoplasm of plasma-cells, deep blue.

D. **Bielschowsky's Silver Stain.**—His method for staining collagen fibrils is the same as for axis cylinders, except that

step 7. the treatment with acetic acid, is omitted. The colla-
gen fibrils are stained intensely black on a clear back-ground.
The method is highly recommended by Herxheimer.

E. **Mall's Differential Method for Reticulum.** — 1. Digest
frozen sections of fresh tissue, 40 to 80μ thick, for twenty-four
hours in the following solution:

> Parke, Davis & Co.'s pancreatin,　　　　5 grams;
> Bicarbonate of sodium,　　　　　　　10 　"
> Water,　　　　　　　　　　　　　100 c.c.

2. Wash carefully in clean water.

3. Place sections in a test-tube half full of water, and shake
thoroughly in order to remove all the cellular débris.

4. Spread out on slide, and allow to dry.

5. Allow a few drops of the following solution to dry on
surface:

> Picric acid,　　　　　　　　　　　10 grams;
> Absolute alcohol,　　　　　　　　　33 c.c.;
> Water,　　　　　　　　　　　　　300 　"

6. Stain for about half an hour in the following solution:

> Acid fuchsin,　　　　　　　　　　10 grams;
> Absolute alcohol,　　　　　　　　33 c.c.;
> Water,　　　　　　　　　　　　66 　"

7. Wash in the picric acid solution for a moment.

8. Alcohol, xylol, xylol colophonium or balsam.

FIBROGLIA FIBRILS

Connective-tissue cells or fibroblasts are characterized by
the production of two kinds of fibrils, fibroglia fibrils, which
bear the same relation to the connective-tissue cells that
neuroglia fibrils bear to glia cells, and collagen fibrils, which
are independent of the cells and occur between them. Fibro-
glia fibrils can be studied to best advantage in actively growing
connective tissue, for example, in chronic salpingitis and in
the stroma of carcinomata; but they are found well developed

in other situations also, as, for example, in the capsules of Pacinian corpuscles.

Fibroglia fibrils can be stained differentially by several different methods. The two simplest and most useful are phosphotungstic-acid hematoxylin and the aniline blue stain after fixation in Zenker's fluid. The fibrils are often stained intensely with eosin, in eosin methylene-blue preparations, if the tissue was perfectly fresh when fixed.

A. **Mallory's Phosphotungstic-acid Hematoxylin Stain.**—Follow the directions given for neuroglia fibrils on page 149.

B. **Mallory's Aniline Blue Stain.**—(See directions on page 118.)

C. **Mallory's Acid Fuchsin Stain.**—1. Fix in Zenker's fluid. The tissue should be as fresh as possible, and cut into thin sections (2 to 4 mm. thick) for the best results.

2. Stain celloidin or paraffin sections in a 1 per cent. aqueous solution of acid fuchsin overnight in the cold, or twenty to thirty minutes in the paraffin oven (56° C.).

3. Wash quickly in water (not over five seconds). Water extracts acid fuchsin very rapidly.

4. Differentiate in a 0.25 per cent. aqueous solution of permanganate of potassium for twenty to forty seconds. This step must not be prolonged beyond the exact time needed or the section will be decolorized.

5. Wash quickly in water (not over five seconds).

6. Dehydrate in alcohol.

7. Clear in xylol.

8. Mount in xylol balsam.

While not an absolutely differential stain for these fibrils, the method, for the most part at least, is perfectly satisfactory. It stains intensely red not only these fibrils and the cell nuclei but also fibrin, the contractile elements of striated muscle-cells, the differentially staining fibrils of smooth muscle-cells, neuroglia fibers, and the cuticular surfaces of epithelial cells. The collagen fibrils of connective tissue cells appear from brownish-yellow to colorless; elastic fibrils, unless degenerated, are bright yellow.

ELASTIC FIBERS

Elastic fibers are not affected by dilute caustic soda or potash, or by acids. These reagents are often used, therefore, to demonstrate elastic fibers in the fresh condition, as, for example, in sputum, because they render them prominent by clearing or destroying the other tissues. The fibers show a marked affinity for osmic acid, staining with greater rapidity than most other tissue-elements.

For bringing out elastic fibers in sections of hardened tissues there are three excellent differential stains. The great advantage of Verhoeff's is that it is applicable after Zenker fixation.

A. **Weigert's Stain for Elastic Fibers.**—Fixation in alcohol or formaldehyde is preferable, but other fixing reagents give good results. Embed in celloidin or paraffin. After fixation in Zenker's fluid, sections stain slowly, and there is a greater tendency, perhaps, to diffuse coloring of the collagen fibrils.

1. Stain sections twenty minutes to one hour in solution prepared as follows:

Fuchsin (basic),	2 gms.;
Resorcin,	4 "
Water,	200 c.c.

Boil the solution in a porcelain dish; when briskly boiling add 25 c.c. of liquor ferri chloridi (a 29 per cent. solution); stir and boil for two to five minutes. A precipitate forms. Cool and filter. The filtrate is thrown away. The precipitate remains on the filter-paper until all the water has drained away or until the precipitate has thoroughly dried. Then return filter and precipitate to the porcelain dish, which should be dry, but which should contain whatever part of the precipitate remained sticking to it. Add 200 c.c. of 95 per cent. alcohol, and boil. Stir constantly, and fish out the filter-paper as the precipitate is dissolved off. Cool; filter; add alcohol to make up the 200 c.c. Add 4 c.c. of hydrochloric acid.

2. Wash off in alcohol.

3. Blot with filter-paper, and add xylol quickly; repeat the blotting, followed by xylol, two or three times until the section is perfectly cleared.

4. Xylol colophonium or balsam.

Sections can be stained for several hours. If the rest of the tissue is overstained, differentiate in acid alcohol; if the sections are too deeply stained, the color cannot be washed out. Diffuse staining can be avoided by diluting the stain either with alcohol or, better still, with alcohol containing 2 per cent. of hydrochloric acid. The elastic fibers appear dark blue, almost black, on a clear background. The nuclei can be stained red with carmine before or after the staining of the fibers. After Zenker fixation, carmine stains are difficult to obtain. A light nuclear stain with alum-hematoxylin, after the fibrils have been colored, is preferable.

The solution keeps for months.

It it be desired to keep sections for some time before mounting, wash them in alcohol and place in water.

B. **Hart's Modification of Weigert's Elastic Tissue Stain.**—

1. Stain sections in lithium carmine thirty minutes.

2. Then direct into

Acid alcohol,	100 c.c.;
Weigert's stain,	5 "

Stain over night; twelve hours at least.

3. Wash in 85 per cent. alcohol; then dehydrate, clear, and mount as in Weigert's method.

C. **Unna's Orcein Method for Elastic Fibers.**—Unna's latest method of using orcein is as follows, and can be highly recommended:

1. Stain sections in the following solution:

Orcein,	1 gm.;
Hydrochloric acid,	1 c.c.;
Absolute alcohol,	100. "

Place the sections in a dish and pour over them enough of the solution to cover them. Warm gently in an incubator or over a small flame for ten to fifteen minutes until the solution

thickens, or leave in the solution at room-temperature overnight.

2. Wash off thoroughly in dilute alcohol (70 per cent.).

3. Wash in water to get rid of all the acid and to fix the color.

4. Alcohol.

5. Oil.

6. Xylol colophonium or balsam.

The washing in water is not absolutely essential.

Elastic fibers are stained of a deep silky-brown color, connective tissue a pale brown. If it is desirable to have only the elastic fibers stained, wash for a few seconds in 1 per cent. hydrochloric-acid alcohol before washing in water. The nuclei can be brought out by staining in Unna's polychrome methylene-blue solution after washing the sections in water.

Verhoeff's Elastic Tissue Stain.—Fixation in formalin or Zenker's fluid preferred. Tissues or sections should not be treated with iodine solution before staining. Mercurial precipitates, if removable, are removed by the staining solution. For the best results the solution should be used within twenty-four hours, but satisfactory specimens may be obtained with solutions one month old.

The staining fluid is made as follows:

Hematoxylin crystals,	1 gm.
Absolute alcohol,	20 c.c.

Dissolve in test-tube by aid of heat, filter, and add in order given:

Aqueous solution (10 per cent.) of ferric chlorid,	8 c.c.
Lugol's solution (iodine, 2; potassium iodid, 4; water, 100),	8 "

Sections are immersed in the staining fluid for fifteen minutes or longer until perfectly black, and are then differentiated in a 2 per cent. aqueous solution of ferric chlorid. The differentiation requires only a few seconds. To observe the stages in the differentiation, the sections may be examined in water under a low magnification. If the differentiation has been

carried too far, the sections may be restrained, provided that they have not been treated with alcohol.

The sections are now washed in water, followed by 95 per cent. alcohol to remove the stain of the Lugol solution, and then are allowed to remain in water five minutes or longer. They are then counterstained in a $\frac{1}{2}$ per cent. aqueous solution of eosin if desired, passed through alcohol, oil of origanum, and mounted in balsam.

By this method elastic tissue is stained black, while connective tissue, fibroglia, myoglia, and neuroglia fibrils, myelin, and fibrin take the eosin stain. Nuclear staining may be obviated by doubling the amount of Lugol's solution in the staining fluid. Degenerated elastic tissue (elacin) is also stained by this method. The degenerated fibrils may be distinguished from the normal by staining less intensely and presenting less distinct outlines.

Equally good results, especially after Zenker's fixation, may be obtained by staining the tissues *en masse*. Myelin, however, is also stained. Thin slices of tissue after fixation are removed from 80 per cent. alcohol and immersed in the staining fluid four days. They are then quickly rinsed in water to remove excess of stain, placed in 80 per cent. alcohol and embedded in the usual manner. The sections are differentiated in a $\frac{1}{2}$ per cent. solution of ferric chlorid.

SMOOTH AND STRIATED MUSCLE=CELLS

For the demonstration of muscle-cells double stains, such as alum-hematoxylin and eosin or eosin and methylene-blue, are sufficient.

For bringing out the finer details in the cytoplasm, however, phosphotungstic-acid hematoxylin and the aniline blue stain are much to be preferred. It is imperative that the tissue be perfectly fresh, especially if the myoglia fibrils in smooth muscle-cells are to be studied, because they very quickly undergo postmortem changes. Thin sections of the tissues to be studied should be put into Zenker's fluid within five to ten minutes at the most after removal from the body, if the best results are

desired. Autopsy material is practically useless. The most desirable tissues are those obtained directly at operations on the human body.

A. Phosphotungstic-acid Hematoxylin Stain (Mallory). For directions see page 149.

B. Aniline Blue Stain (Mallory). (See page 118.)

C. **Benda's Stain for Myoglia Fibrils.**—1. Fix fresh material in Zenker's fluid for twenty-four hours.

2. Wash for a number of hours in water.

3. Make frozen sections.

4. Place sections in a 0.5 per cent. solution of chromic acid for twenty-four hours.

5. Wash off in water.

6. Place in a 0.25 per cent. solution of permanganate of potassium for about three minutes.

7. Wash off in water.

8. Place in Pal's mixture of sulphite of sodium and oxalic acid for five minutes.

9. Wash off in water; take up section on slide.

10. Cover with the following solution:

Crystal-violet, saturated solution in 70 per

cent. alcohol,	1 part;
Acid alcohol,	1 "
Aniline-water,	2 parts.

11. Blot with filter-paper.

12. Cover with dilute Lugol's solution.

13. Blot with filter-paper; dry.

14. Differentiate in aniline oil and xylol, equal parts.

15. Xylol; xylol balsam.

THE CENTRAL NERVOUS SYSTEM[1]

The choice of a method of preservation of the central nervous system is governed by the kind of investigation to be attempted. For gross anatomical studies and the coarser microscopic work, *i.e.*, tract degeneration, fixation of the whole brain without

[1] This section has been prepared by Dr. Samuel T. Orton.

sectioning is recommended. For this purpose formalin (4 per cent. formaldehyde gas) has practically entirely superseded the older method of fixation in chrome salt solutions. The brain has a slightly greater specific gravity than the formalin solution and hence will sink in the fixing jar and suffer distortion by flattening. This difference in specific gravity is so slight, however, that a thread slipped under the basal vessels will suspend the brain without undue strain on the vessels and consequently with very little distortion except in the extremely soft brains of very young children and of cases dying from acute infections. The formaldehyde should be changed after three or four days and again after two or three weeks. It requires two or three weeks of fixation so to harden a brain that gross slabs can be cut of any desired thickness, preferably about 1 cm., for orientation of deep gross lesions and these may then be cut for finer histological work. Material so fixed, however, is not available for some of the best methods for demonstrating special structures. Some of these will not give their best results after even a short fixation period in formalin and there are several which will not work to advantage after the two or three weeks fixation required for whole brains. When, therefore, the finer histological changes are to be studied, immediate sectioning of the fresh brain and direct fixation in from three to five different fixatives are recommended.

The selection of the areas from which these small pieces are to be cut is important, not only from the standpoint of correlation of locus with the clinical picture but often from the standpoint of diagnosis as well, as, for example, in general paresis where the lesions in the field of distribution of the posterior cerebral artery, *i.e.*, the occipital lobes, are frequently so mild as to make the establishment of the diagnosis difficult or actually impossible, while in the frontal regions, especially over the convexity, the lesions are usually advanced. Where focal gross lesions exist, of course, pieces should be taken from both the lesions and the adjacent fields but often no gross lesions are present and in such brains a fairly wide sampling is recommended.

A choice of areas which has gained favor in a number of laboratories is as follows:

1. Precentral gyrus (motor area) high up on the convexity near the inter-hemispheric cleft. This is the area controlling the lower trunk and thigh muscles and the Betz cells here are of large size. 2. Postcentral gyrus immediately back of the motor samples. This is the area subserving skin, joint and muscle senses. 3. Frontal. Pieces from the inter-hemispheric margin about midway between the frontal pole and the point of incidence of the Rolandic fissure. This is the so-called anterior association zone. 4. Temporal. From the exposed surface of the first temporal gyrus just below the Sylvian fissure and opposite the lower end of the fissure of Rolando. This is auditory cortex. 5. Occipital. From the occipital pole adjacent to the calcarine sulcus and hence representing the area striata which can be identified with the naked eye because of the prominent white line parallel to the cortex surface and about mid-way in its depth. This is the "arrival platform" subserving vision. 6. Hippocampal cortex. These blocks are taken from the inner edge of the temporal lobe opposite the crura cerebri and represent an area of olfactory function.

These areas taken from one hemisphere give a satisfactory routine survey although it is frequently advisable to take the same series from both sides. In order to preserve these sections so that their source may be recognized later they may be labeled in an effective and simple manner with bits of filter paper. Small pieces of filter paper are cut and marked with a name or symbol in lead pencil and pressed lightly on the fresh cut surface of the block of brain tissue. As soon as the filter paper has imbibed some of the coagulable material from the cut surface the block is carefully immersed in the fixative. Coagulation fixes it firmly. Tags so applied stick well after alcohol and Zenker fixation, and in practically all fixatives they adhere well enough to stay in place if the material be handled with some care. If both hemispheres are cut, square tags can be applied for the right hemisphere and triangular ones for the left.

For the preservation of these blocks the following fixation methods, arranged more or less in the order of their importance, are recommended.

Formalin.—(See page 40.) In addition to its use as preservative for whole brains, formalin is widely used for the fixation of small pieces. In some procedures the period of immersion is not important but in others the formalin serves only as a preliminary fixative and the material must be carried on into mordanting solutions within 24 to 48 hours after fixation starts. If only one fixative is to be employed, formalin is unquestionably the best because of its wide range of usefulness. It is probably, however, the most variable in its results of any of the standard fixatives. During the early stages of fixation the nervous tissues swell considerably in formalin and then gradually undergo a shrinkage leading often to marked distortion of the cells and other delicate structures. The fixation produced by formalin is apparently not firm enough to withstand later dehydration with alcohol without marked shrinkage distortion, so that material so fixed does not give good paraffin sections. Further mordanting, as for example in preparing formalin material for the Weigert medullary sheath stain, will often overcome some of this difficulty. Some methods such as the Bielchowsky and the Weigert can be successfully applied after several years in formalin if care is taken to wash the tissue thoroughly. Even with short fixation it is advisable to wash overnight in running tap water before further mordanting or embedding and with very old tissues washing for 48 or 72 hours is recommended.

Alcohol.—For the study of the extranuclear chromophile particles of the nerve cells (the Nissl or tigroid masses) the best fixative is 95 per cent. alcohol. Even when relatively small pieces are preserved the alcohol should be in the proportion of about 10 to 20 times the volume of the tissue and should be changed daily for at least a week. If larger pieces are used, the changes should be continued on alternate days through the second week and for two or three times during the third week. After such hardening blocks may be kept indefinitely in alcohol

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and embedded when wanted. Blocks so fixed give excellent pictures after 8 to 10 years.

Zenker's Fluid.—(See page 44.) Zenker's fluid is one of the most valuable fixatives for brain and cord material. Fix as directed on page 45 except that better results will be obtained in brain material by dehydrating in graded alcohols, instead of directly in 80 per cent. alcohol as there advised. Material so fixed may be preserved for years and may be used for the study of the fibrous neuroglia after the phosphotungstic acid hematoxylin stain and for the study of the vessels, meninges, inflammatory exudates and many tumors after the eosin-methylene blue stain (page 100) and Verhoeff's elastic tissue stain (page 124).

Weigert's Glia Mordant.—This mixture used after preliminary fixation for 24 hours in formalin or better used with formalin added during the first 24 hours is valuable in conjunction with Alzheimer's modification of the Mann eosin-methyl blue stain for demonstrating the cytoplasmic neuroglia, especially the so-called ameboid forms of neuroglia cells. This material is also available for the Weigert stain for the fibrous glia. The fixing fluid is the fluorchrome copper acetate and acetic acid mixture given as the secondary mordant under the Weigert method for medullary sheaths and the formula and method of preparation are given there. For fixation of fresh tissues one part of 40 per cent. formaldehyde is added to 8 parts of glia mordant and tissues are placed in this for 24 hours and then transferred to fresh solution to which no formalin has been added. Daily changes of the mordant for the first week are recommended. The best results are obtained in tissues sectioned shortly after this mordanting period but good stains can usually be obtained after several months to a year in the mordant.

Müller's Fluid.—(See page 46.) Müller's fluid as a primary fixative is no longer used as freely as formerly. It is valuable, however, in the fixation of tissue for the Marchi method. This method is frequently employed after formalin fixation but the results are by no means so satisfactory. Müller's fluid hardens

slowly and frequent renewal during the fixation period is necessary. Fresh changes daily during the first week, every other day during the second week and twice during the third week are recommended. Material so fixed is excellent for Weigert preparations as well as for the Marchi method.

The staining of the various histological elements of the nervous system and the fixing reagents best suited for each of them will be considered under the following headings:

1. *General Stains:*

2. *Stains for the Tissues of Mesodermal Origin.*—These are employed for the study of the meninges, blood vessels, proliferative and exudative inflammatory reactions and many tumors.

3. *Nerve Cell Stains:*
   A. Cytoplasmic granules. The Nissl or tigroid bodies.
   B. Fibrillar structures including the intra-cellular neurofibrils, the axis cylinders, the dendrites and certain terminal structures such as the muscle plates, etc.
   C. Medullary sheaths.

4. *Neuroglia Stains:*
   A. Fibrous neuroglia including the normal fibrous reticulum and neuroglial scars.
   B. Cytoplasmic neuroglia including the early stages of neuroglia cell reduplication and the ameboid neuroglia cells.

5. *Degeneration Products:*
   A. Fat stains.
   B. Other degeneration products.

I. **General Stains.**—General stains include the ordinary nuclear stains with or without a counterstain and certain diffuse stains which color practically all elements of the nerve tissue but with slight variations of intensity or shade so that individual structures can be identified. The best fixation for general stains is Zenker's fluid to be followed by eosin-methylene blue. After other fixatives, especially after fixation for a long time in formalin, alum-hematoxylin or Weigert's iron hematoxylin as

nuclear stains followed by either eosin or preferably Van
Gieson's picro-acid fuchsin as a counterstain, give fair results.

A: **Van Gieson's Stain.**—This may be used after practically
any fixation. Freeborn's exact formula is preferable to the
original method:

<div style="padding-left:2em">

1 per cent. aqueous solution of acid fuchsin,   15 c.c.;
Saturated aqueous solution of picric acid,   50 "
Water,   50 "

</div>

1. Stain sections rather deeply in alum-hematoxylin or Wei-
gert's iron hematoxylin.
2. Wash in water.
3. Stain in the above solution three to five minutes.
4. Dehydrate in alcohol.
5. Clear in oil or xylol.
6. Xylol balsam.

The nuclei are deep blue to bluish red depending on the depth
of the hematoxylin stain, ganglion cell bodies and their coarser
processes red, axis cylinders darker red, medullary sheaths
pale yellow, neuroglia and connective tissue fibers deep red,
the bodies of the ameboid glia cells and the Füllkörperchen are
pale red to pink. With some material the Van Gieson mixture
stains weakly. This may often be overcome by staining in
the incubator for one-half to one hour or the strength of the
mixture may be increased by omiting the water wholly or in part.

B. **Phosphotungstic acid hematoxylin** (see page 72) is
valuable as a general stain. The method is given under stains
for fibrous neuroglia.

C. **Phosphomolybdic Acid Hematoxylin.**—(See page 71.)
This solution stains well after fixation in Müller's fluid or in
frozen sections after fixation in Weigert's glia mordant. The
method of application for the latter material is given under the
plasmatic neuroglia. After Müller's fluid material may be
embedded in either celloidin or paraffin.

1. Stain sections twenty minutes to one hour.
2. Wash out in several changes of 50 per cent. alcohol, until
the color no longer comes out in clouds.

3. Dehydrate in 95 per cent. alcohol.
4. Oil. Xylol. Xylol balsam.

The ganglion cells are deeply stained. The axis cylinders and neuroglia fibers are stained to about the same intensity.

II. **Stains for the Tissues of Mesodermal Origin.**—In addition to the general stains given above certain special stains are often of value for demonstrating alterations of the meninges and vessel walls and for the study of exudative cells, etc. Zenker's fluid is the most valuable fixative for this purpose, and may be followed by the eosin-methylene blue method (page 100) which serves for a general review of these tissues and at the same time gives good general pictures of the nerve tissue. Good preparations by this method also show the Nissl bodies in the nerve cells to very good advantage, although such preparations are not to be compared with the results obtained by the toluidin blue method. The medullary sheaths take a faint pink fairly homogeneous color by this method while degenerating sheaths are more deeply colored and appear very finely granular and somewhat shrunken. Moreover, dense neuroglia scars stain deep pink to red so that preparations by this method will serve for preliminary orientation though the special stains are recommended for further details of these processes. Material fixed in Zenker's fluid may also be stained by the anilin blue connective tissue stain (page 118) and by Verhoeff's elastic tissue stain (page 124). For the demonstration of the plasma cell exudate in paresis two special methods are valuable. The first is the toluidin blue method for staining the Nissl bodies and is given under that heading. The other is the Unna-Pappenheim pyronin-methyl green stain (page 117). Both of these work to best advantage after fixation in 95 per cent. alcohol. The pyronin-methyl green stain is less dependable than the toluidin blue but gives a contrast differentiation as the cytoplasm of the plasma cell is stained red and its nucleus green.

III. **Nerve Cell Stains.**—1. **Stains for the Nissl or Tigroid Bodies.**—The original Nissl method is used very little today because of the difficulty of cutting the unembedded blocks to accurate thickness and of handling the sections through the

staining and mounting processes. It has been largely super-
seded by methods which permit of embedding and of these the
toluidin blue can be recommended most highly both as to
dependability, simplicity and permanence. The method is
given according to Alzheimer's directions.

A. 1. Fix in 95 per cent. alcohol.

2. Embed in celloidin and cut.

3. Transfer sections to a 0.25 per cent. aqueous solution
of toluidin blue in a porcelain evaporating dish. Heat to
steaming, withdraw the flame and leave for 10–15 minutes.

4. Rinse in water.

5. Decolorize in 95 per cent. alcohol. Control decoloriza-
tion under the microscope. The background should be colorless
or very pale blue.

6. Oil. Xylol. Xylol balsam.

The toluidin blue stock solution (0.25 per cent. aqueous) keeps
well and improves somewhat with age. It should be filtered
into the evaporating dish for use to avoid precipitate. With a
ripened solution the Nissl bodies are strikingly stained and show
a purplish color which is n sharp contrast with the dark blue of
the nucleus. This same contrast is evident in the plasma cells
where the cytoplasm takes the purple color and the nucleus blue.
The apical process and the larger basal processes are faintly
stained for a short distance but the axis cylinders, medullary
sheaths and glia fibers are unstained giving a clear background.
Glia cell nuclei are stained deep blue. The cytoplasm of glia
cells is pale blue. When ameboid glia cells are present in
numbers their cytoplasm is bluer than that of the nerve cells
and plasma cells but the depth of stain is variable, some are
very pale and others—even adjacent ones—are dark blue.

Kresyl violet, thionin or Nissl's soapy methylene blue may
be substituted for the toluidin blue in this method in the same
strength but they do not give quite so sharp a metachromatic
differentiation as does toluidin blue.

Formalin fixed material may be stained by this method or
after embedding in paraffin. Thionin and kresyl violet give

slightly better results after formalin than does toluidin blue, but none of them compares favorably with the alcohol toluidin blue method. Alcohol seems to act as a better coagulant of the tigroid body material than either formalin or Zenker's fluid and the masses appear larger, more sharply defined and in a clearer matrix after such fixation. In old formalin material the whole cell body is frequently intensely stained so that the details of the Nissl bodies cannot be well made out and often the intercellular background also stains deeply so that in differentiation most of the cell detail is lost before the background clears up enough for detailed study.

2. **Fibrillar Structures.**—They include all of the fibrillar mechanism of the cell body and its expansions, *i.e.*, that part of the neurone which is looked upon as the transmitting mechanism:—the intra-cellular neurofibrills, the dentrites, the axis cylinder and its arborization and often its terminal structures. With few exceptions the methods employed for the demonstration of these structures depend on impregnation with a metal salt and later reduction or development within the tissues. All of the impregnation methods are strikingly variable in their results especially when applied to human material with a variable postmortem period before fixation. In some structures it is also probable that the metallic deposit is in the nature of a surface crustation rather than of incorporation of the metal with the tissue element and distortions both of size and relation may enter from this fact. The classical Golgi methods while still in use occasionally have been largely superseded by others. The method in most common use on pathological material is the Bielchowsky. Bielchowsky has published two methods, one for sections and one for blocks, but the many failures of infiltration and the great variability of results in the block method have brought it into more or less disfavor.

A. Bielchowsky's method for sections:

    1. Fix in formalin 24 hours or longer.
    2. Wash freshly fixed material thoroughly in running water for 2 to 5 hours, old material for 24 to 48 hours.

3. Cut frozen sections as thin as possible, preferably under 10μ.

4. Rinse in two or more changes of distilled water. Handle with glass needles from here on.

5. Transfer to pure pyridin for 24 hours, and wash again thoroughly in distilled water. This step is an addition to the earlier formula and may be omitted.

6. Impregnate in a 2 or 3 per cent. aqueous solution of silver nitrate for 24 hours in the dark.

7. Carry through distilled water and into the following alkaline silver bath for 10 to 20 minutes or until the sections become a dark seal brown:

To 5 c.c. of a 10 per cent. aqueous solution of silver nitrate add 5 drops of freshly prepared 40 per cent. aqueous solution of sodium hydrate. The precipitate so produced is redissolved by the addition of ammonium hydrate drop by drop with shaking. The solution is then diluted to 20 c.c. with distilled water and is ready for use. (Great care in the use of pure water and pure chemicals and clean glassware is necessary. The amount of ammonia added to dissolve the precipitate should never exceed 18 to 20 drops. If more is needed the results are rarely satisfactory. Washing the precipitate, before dissolving, in several changes of distilled water by decanting either after sedimenting or centrifuging often helps in the preparation of a satisfactory bath.)

8. Wash in distilled water.

9. Reduce for 12 to 24 hours in an 8 per cent. aqueous solution of formaldehyde (20 per cent. formalin).

10. Rinse in distilled water and tone in the following solution for 10 to 20 minutes or until the ground color is a reddish violet.

2 to 3 drops of 1 per cent. solution of gold chlorid are added to 10 c.c. of distilled water and to this are added 2 to 3 drops of glacial acetic acid.

11. Rinse in distilled water and fix $\frac{1}{2}$ minute in 5 per cent. thiosulphite of sodium (photographer's "hypo" of commercial purity does well) to which 1 drop of sodium bisulphite may be added.

12. Rinse thoroughly in distilled water to remove the hypo.

13. Dehydrate in 95 per cent. and absolute alcohol. Clear in carbol-xylol (1 part carbolic acid to 9 of xylol). Mount in xylol balsam.

This method serves as an excellent one to stain axis cylinders and dendrites, and in well stained thin sections the intracellular neurofibril net will be seen as a delicate mesh work, of dark brown or black lines. The axis cylinders and their processes and the muscle plate termini in striped muscle are stained intensely black. The glia fibrils are very faintly stained and can be seen only with difficulty except where there has been marked proliferation as in the glia scars in tabes or multiple sclerosis. Here they can be seen as fine, usually closely packed, wavy lines colored a delicate lavender or purple. Longitudinal sections of the cord in multiple sclerosis stained by this method show to excellent advantage the axis cylinders intact but with their medullary sheaths replaced by fibrous glia.

B. *Ranson's Pyridin Silver Stain.*—1. Fix for 48 hours in absolute alcohol to which 1 per cent. ammonia has been added (95 per cent. alcohol with 5 per cent. ammonia may be used).

2. Wash from $\frac{1}{2}$ to 3 minutes (according to size) in distilled water.

3. Pyridin for 24 hours.

4. Wash in many changes of distilled water for 24 hours.

5. Impregnate for 3 days in the dark in a 2 per cent. aqueous solution of silver nitrate at 35° C.

6. Rinse in distilled water and reduce in a 4 per cent. solution of pyrogallic acid in 5 per cent. formalin.

7. Cut paraffin sections which are ready for examination after mounting.

This method is said to give good results in the peripheral nerves, to be applicable to larger pieces with uniform impregnation and to be much more reliable than the other silver methods. It also stains the non-medullated fibers more intensely than others thus serving as a differential stain for them. Huber and Guild applied the method to decalcified material thus enabling sections of the entire head of embryos and small an'mals to be made.

C. Methylene Blue Stain for Nerve Fibers.—This method originated with Ehrlich. It is used chiefly in the study of the peripheral nerves particularly in their distribution in tissues and organs, although it is also applicable to the central nervous tissues. Its use has been largely restricted to the study of normal material. Many modifications of the original procedure have been suggested with a view to making the results surer or the specimens more permanent. Tissues can be stained either by injection or by immersion.

The methylene-blue used should be "rectified methylene blue for vital injection."

For injection in the blood- or lymph-vessels of live or dead animals a 1 to 4 per cent. solution in normal salt solution is recommended. The injected organs are exposed to the air until a bluish tint is visible. As soon as the greatest intensity of stain is reached (five minutes to two hours) the color in the preparation is fixed by placing small bits of the tissue in a freshly filtered, cold, saturated, aqueous solution of picrate of ammonium, or better still, in the solution given below, recommended by Bethe.

Very small or thin pieces of tissue intended for staining by immersion (the method employed for human tissues) are placed in a very dilute solution ($\frac{1}{60}-\frac{1}{15}$ per cent.) of methylene blue in normal salt solution.

The stain may then be fixed by the method already given, or better still, in the following manner:

Bethe's method of fixing methylene blue stains of nerve-fibers:

1. Wash off excess of color with normal salt solution.
2. Place in:

Molybdate of ammonium,	1 gm.;
Distilled water,	10 c.c.;
0.5 per cent. osmic acid,	10 "
Hydrochloric acid,	1 drop.

A precipitate forms on making up the solution, but disappears on shaking. The solution is best made up fresh each time. It should be used as cold as possible, preferably surrounded by a mixture of ice and salt. Leave the tissue in the cold solution for from two to five hours, and then for a while longer at room temperature.

3. Wash one half to two hours in running water.
4. Dehydrate and harden as quickly as possible (not over twelve to twenty-four hours) in cold absolute alcohol. (The color is soluble in warm alcohol.)
5. Clear in xylol.
6. Embed in paraffin.

The sections may be mounted directly or brought into water and stained with alum-cochineal for contrast.

Many of the phosphotungstic and phosphomolybdic acid preparations also serve as axis cylinder stains in the white matter of the cord and brain. They are not, however, as selective as the silver stains. Alzheimer's modification of Mann's eosin-methyl blue method gives excellent pictures of the axis cylinders and affords a selective differentiation of the degenerating from the normal before changes are demonstrable by the Marchi method in the medullary sheaths. In cross sections of the white matter of the cord the normal axis cylinders are deep purple or blue while those undergoing degeneration are shining red. This change can be demonstrated in the experimental animal within 48 hours after damage to the cord. This method is given in detail under the stains for the plasmatic glia.

3. **Stains for the Medullary Sheath.**—The medullary sheaths are probably complex mixtures of lipoids. These lipoids will

reduce osmic acid to metallic osmium and the earlier methods of staining the sheaths depended on this reaction. These have been discarded, however, because of their expense and the fact that osmic acid does not penetrate well.

Weigert originated the differential hematoxylin stain which in some of its modifications is universally used as a sheath stain today. This depends on mordanting with a chrome salt which combines with the lipoids of the sheaths in such a way as to render them insoluble in the embedding reagents and also to prepare them for the stain or for further mordanting. This combination of a chrome salt with the medullary sheaths also so alters them that they will no longer reduce osmic acid while the fats produced by their degeneration are not so changed and this serves as a bàsis of the Marchi method for the demonstration of degenerating medullary sheaths. Weigert further mordanted this chromated material with a copper salt which served to bind the copper to the chromated sheaths, with the resultant formation in the staining fluid of a copper lake of hematoxylin which is resistant to the decolorizing action of certain differentiating fluids. Weigert later recommended an iron-hematoxylin which gives a stain somewhat more resistant to the differentiating solutions. In the earlier methods the mordanting process was slow, the background remained yellow or yellow brown in completed preparations and the differentiating fluid acted so vigorously that there was danger of completely decolorizing some of the finer sheaths of the cortex. Many modifications of the original method were offered. Weigert himself offered a mordant containing two chrome salts ("Schnellbeize") which reduces very materially the time required in the original procedure for mordanting the sheaths. Of the others the most commonly used are the Pal, which aims at clearing the background by bleaching with potassium permanganate and oxalic acid, the Kulschitsky, which gives a very slow differentiation and hence less danger of decolorization of the finer fibers, and the Wolters modification of Kulschitsky which combines with the latter the Pal method of bleaching the background.

The choice between these various methods depends on the purpose for which the material is to be used. The Weigert or the Weigert-Pal are advised where the coarser mass degenerations, as, for example, the tabetic degeneration of the posterior columns, are to be demonstrated and where the finer, more delicate sheaths of the gray matter are not considered of consequence. Weigert-Pal sections give clear cut pictures, permit the use of counterstains such as the van Gieson and are much to be preferred for projection and naked eye demonstration specimens. Their decolorization is so drastic, however, that many of the finer sheaths are bleached and they are not advised for more careful study. For this purpose the Kulschitzky stain is most highly recommended. The Wolters method gives clearer pictures but partakes of the dangers of the Pal. In the order of their dependability for staining the more delicate fiber sheaths these four methods probably rank as follows: Kulschitzky, original Weigert, Wolters, and Pal. In the Kulschitzky method the staining is done in an acid hematoxylin solution and the formation of the lake is carried out at the same time as the differentiation by combining the lithium carbonate with the potassium ferricyanide.

Embedding should be in celloidin. Thin sections are not desirable except for the finer studies in the cortex. The medullary sheath stains give negative pictures of defects, *i.e.*, it is the normal sheaths which are stained and the loss of an occasional fiber cannot be detected. The method is, therefore, most applicable to coarse loss of groups of fibers as in tract degenerations, and thick sections give much better contrast with these areas of defect than do the thinner ones. The usual thickness for cortex sections is 30μ. Cord sections are often better at 20μ.

The sheaths stain for some time after degeneration starts and it is not uncommon to find masses of degenerated sheath material, which still takes the characteristic stain, engulfed in phagocytes. Because of this fact and the very slow absorption of degenerated sheaths the Weigert method and its modifications do not give true pictures of the amount of fiber loss for two or three months after the occurrence of the lesion.

For total brain sections the Kulschitzky method is recom-
mended.   The brain is first fixed in toto in formalin for three
weeks.   Slabs not over 2 cm. thick are then cut and put into
Müller's fluid for 10 weeks or more with frequent changes.   The
use of Weigert's double chrome mordant (the first mordant)
is not recommended.   It acts more quickly and gives more
consistent pictures of the finer sheaths, but it does not penetrate
so well and renders the tissues more brittle, and as these total
sections are used chiefly for the topographical survey of coarse
lesions the minor losses in fine fibrils are not a detriment.   After
careful dehydration the slabs are carried into thin celloidin
(through absolute alcohol and ether as usual) and left in this
for 6 to 8 weeks in closed vessels and then for about a week in
thick celloidin.   After this the celloidin is allowed to harden
by slow evaporation of the solvents and the block mounted and
cut.   Sections are carried through the stain between sheets
of toilet paper.

Two methods are given for staining the medullary sheaths in
frozen sections.   In both of these the stain is an iron-hematoxy-
lin and the mordanting is done on sections and is comparatively
very brief.   They have the advantage of rapidity and are
accurate but they have also the disadvantages of the frozen
section method.

A. Weigert's Method.—1. Fix in formalin or in formalin
followed by Müller's fluid.   The latter method gives excellent
pictures but as the material ages it gets progressively more
brittle.   Formalin material of almost any age gives good coarse
pictures but in old material the finer sheaths often fail to stain.

2. Primary mordanting.   Small blocks not over 1 cm.
thick are placed for 4 to 5 days at room temperature or 2 to 3
days at 37° C. in the following solution (known as Weigert's
first mordant, or "Schnellbeize"):

Bichromate of potassium,	5 gms.;
Fluorchrome,	2 "
Water,	to 100 c.c.

3. Secondary mordanting. (This is Weigert's glia mordant, the Glia Beize or Kupfer Beize of the German writers.) Transfer tissues directly to the following solution for 24 to 48 hours:

Acetate of copper,	5.0 gms.;
Acetic acid (36 per cent.),	5.0 c.c.;
Fluorchrome,	2.5 gms.;
Water,	to 100.0 c.c.

The fluorchrome is dissolved by boiling the water, the flame is withdrawn and the acetic acid added as soon as boiling has ceased and then the finely powdered copper acetate is added and stirred vigorously until dissolved. This mordant may be applied to either blocks or sections. If desired the blocks may be embedded in celloidin after the first mordant. Both procedures give good results.

4. Dehydrate in graded alcohols beginning with 50 per cent. and with no steps greater than 10 per cent. to avoid shrinkage. Embed in celloidin and cut at 20 or 30μ.

5. Stain in the following solution for 24 hours:

a. Liquor ferri chloridi (officinal),	4 c.c.;
Water,	96 "
b. Ripened 10 per cent. solution of hematoxylin in absolute alcohol,	10 "
95 per cent. alcohol,	90 "

A mixture of equal parts of these two solutions blackens immediately through the formation of iron-hematoxylin and forms the staining fluid. Keep in stock a 10 per cent. solution of hematoxylin in absolute alcohol. At least ten days of exposure to light are required to ripen this solution and it does not reach its greatest staining strength for several months.

The earlier staining fluid gives good results but is not quite so resistant to decolorization by the differentiating fluid. It is as follows:

Ripened 10 per cent. solution of hematoxylin in absolute alcohol,	10 c.c.
Saturated aqueous solution of lithium carbonate,	1 "
Water,	90 "

A saturated solution of lithium carbonate should be kept on hand as the salt dissolves rather slowly.   Mix only the amount required for immediate use.

6. Rinse and differentiate in the following solution:

Borax,	2 gms.;
Ferricyanide of potassium,	2.5 "
Distilled water,	100 c.c.

Differentiation is complete within a half hour, sometimes within a few minutes.   If the differentiation takes place too rapidly the fluid may be diluted one-half.

7. Wash thoroughly to stop the decolorizing.   Dehydrate in alcohol, clear in aniline oil-xylol or carbol-xylol and mount in xylol balsam.

The iron hematoxylin gives black sheaths on a pale yellow or almost colorless background, the lithium preparations give blue sheaths on a dark yellow or brown field.

B. Weigert-Pal Method.—1. Fixation and primary mordanting as for Weigert's method.

2. Place sections for several hours in a 0.5 per cent. aqueous solution of chromic acid, or for a longer time in a 2 to 3 per cent. solution of bichromate of potassium.   This step is often omitted, especially when the tissues have been but recently mordanted.

3. Transfer sections to Weigert's alcoholic hematoxylin solution for twenty-four to forty-eight hours (if necessary for an hour in the incubator at 37° C.).

This solution is as follows:

Ripened 10 per cent. solution of hematoxylin, in absolute alcohol,	10 c.c.;
Water,	90 "

4. Wash in water plus 1 to 3 per cent. of saturated aqueous solution of carbonate of lithium until the sections appear of a uniform deep-blue color.

5. Differentiate for twenty seconds to five minutes in a $\frac{1}{2}$ per cent. aqueous solution of permanganate of potassium until the gray matter looks brownish-yellow.

6. Transfer to the following solution:

Oxalic acid,	1 gm.;
Sulphite of potassium,	1 "
Water,	200 c.c.

for a few seconds until the gray matter is colorless or nearly so.

7. Wash thoroughly in water.
8. Dehydrate in 95 per cent. alcohol.
9. Oil, xylol balsam.

Steps 5 and 6 sometimes have to be repeated when the differentiation has not been complete.

This method gives excellent pictures for low power examination and projections but is not so trustworthy for finer studies.

C. Kulschitzky's Modification of the Weigert Stain.—1. Fixation and primary mordanting as for Weigert's method:

2. Dehydrate in alcohol and embed in celloidin.
3. Stain sections for 24 hours in the following solution.

10 per cent. solution of hematoxylin in absolute alcohol,	10 c.c.;
2 per cent. acetic acid,	90 "

4. Carry over directly into the differentiating fluid:

Saturated aqueous solution of lithium carbonate,	100 c.c.;
1 per cent. solution of potassium ferricyanide,	10 "

5. Wash thoroughly in water.
6. Alcohol, oil, xylol, xylol balsam.

Differentiation is very slow by this method and can be readily controlled under the microscope. Further, the fact that the formation of the hematoxylin lake takes place in the differentiating fluid minimizes the danger of decolorizing the finer sheaths. This is the recommended method for finer studies of cortical architectonics and for total brain sections.

D. Kulschitzky-Wolters' Modification of the Weigert Method.—Steps 1, 2 and 3 are the same as in the Kulschitzky method.

4. Dip sections in Müller's fluid.
5. Differentiate in permanganate of potassium followed by water and the oxalic acid solution as for the Pal method.

10

6. Wash thoroughly in water to which some ammonium chlorid is added.

7. Alcohol, oil, xylol, xylol balsam.

E. Wright's Method for Frozen Sections.—1. Fix in 4 per cent. formaldehyde solution.

2. Cut frozen sections.

3. Attach the section to the slide by the *aniline-clove oil method* (see page 54).

4. Cover the section with 10 per cent. aqueous solution of ferric chlorid for five minutes.

5. Remove excess of ferric chlorid solution from around the section, leaving some on the section, and pour on the slide as much as it will hold of a freshly prepared aqueous solution of hematoxylin. Let stand for thirty minutes. The hematoxylin solution is prepared by heating a few crystals of hematoxylin in about 15 c.c. of distilled water. The hematoxylin must be of good quality.

6. Wash quickly in water.

7. Differentiate by moving the slide about in 10 per cent. aqueous solution of ferric chlorid, until the gray substance is defined and the pia appears white.

The attachment of the section to the slide by the method specified is important, because it prevents distortion of the section, and because the treatment of the section facilitates the staining, apparently by the removal of interfering fatty material.

8. Wash *thoroughly* in a large quantity of water.

9. Dehydrate in alcohol.

10. Clear in origanum oil.

11. Mount in xylol colophonium or balsam.

F. Spielmeyer's Method for Frozen Sections.—1. Fix . in formalin.

2. Wash in running water.

3. Frozen sections 20 to 30μ thick.

4. Place for 6 hours in 2.5 per cent. aqueous solution of iron-ammonium sulphate (iron alum).

5. Rinse in water and then place for 10 minutes in 70 per cent. alcohol.

6. Stain 10 to 24 hours in the following solution:

10 per cent. solution of hematoxylin in absolute
    alcohol (well ripened),   5 c.c.;
Distilled water,   100 "

7. Rinse and differentiate in the iron alum solution.   Control under the microscope.

8. Wash thoroughly, dehydrate in alcohol, xylol, xylol balsam.   The hematoxylin solution must be old and should be used over and over again as its staining power improves with age.

The washing in 70 per cent. alcohol is to remove fat substances which might interfere with even staining.   Occasional, irregular staining indicates that the fat has not been entirely removed.   Such sections may be carried back through water to 70 per cent. alcohol and again stained and differentiated.

IV. **Neuroglia Stains.**—1. **Stains for the Fibrous Neuroglia.**—The older conception of the neuroglia was gained largely through the application of the Golgi silver methods and the pictures obtained of the relation of the neuroglia fibrils to the cell bodies was misleading in that the incrustation led directly over from the fiber to the plasmatic extensions of the angular cell body and gave a picture of simple elongation of the cytoplasm into fibrillar expansions.   The later methods, particularly Mallory's phosphotungstic acid hematoxylin and Weigert's stain, color the fibrils selectively and have led to the present conception of the fiber as a differentiated structure which, in the mature state, lies in contact with the parent cell or may even be free.   For good pictures of the fibrous neuroglia fresh tissue is of the utmost importance.   The best results are obtained on material taken from the body within one hour after death.   After four to six hours the results are only fair and after 24 hours the loss in normal neuroglia fibrils is often marked.   All fibrils do not, however, disappear with equal rapidity after death.   The coarse fibrils of neuroglia scars and the heavy fibers of the white matter of the cord and those arising from the buried ends of the ependymal cells apparently

resist disintegration longer than the finer supportive framework
of the cortex and white matter of the cerebrum.   Furthermore
this disintegration is not entirely a postmortem change.   In
cases of death following high febrile periods often the neuroglia
stains fail practically entirely even on fresh material.   Alz-
heimer has demonstrated the disintegration of the fibers in
such cases and this destruction is probably to be correlated
with the "soft brains" of acute infectious diseases.   Here also
the destruction apparently is more striking in the finer normal
fibers than in coarser network of glial replacements.

For good results by either the Mallory or the Weigert method,
it is important to take small pieces of the fresh tissue.   With
phosphotungstic acid hematoxylin the fibers are not so intensely
stained so that the picture is not so striking but while more
delicate, the differential effect is equally good.   This process
also has the advantage that it is much simpler and that after
fixation the material is permanent and sections can be prepared
as needed.   With the Weigert method practically all the steps
must be carried out without delay in order to get the best results.
Even after staining there is greater danger of fading in the Wei-
gert stain.   This is particularly true if the aniline oil has not
been thoroughly removed or if the finished sections are exposed
to escaping illuminating gas (Homburger).   Material fixed
in Zenker's fluid and hence prepared for the phosphotungstic
acid hematoxylin method is also available for a variety of gen-
eral stains and for study of the mesodermal elements in the
nervous tissues.   Material fixed in Weigert's glia mordant is
available for the demonstration of the plasmatic neuroglia by
several methods.   Where striking contrast is desired as for
photomicrography and demonstrations to students the Weigert
method is recommended.   However, the comparative simplicity,
the permanence of the stain and the greater delicacy of the
pictures without sacrifice of selectivity make Mallory's
phosphotungstic acid hematoxylin the method of choice for
practically all purposes.   Both methods stain fibrin intensely
and also the fibroglia and myoglia fibrils, when these are
freshly fixed.

Most neuroglia methods work to much better advantage in the white matter than in the gray. In the white matter of both cord and brain the clear background furnished by the practically colorless medullary sheaths serves to bring out the neuroglia cells and fibers with great distinctness. In the cortex and spinal gray matter, however, the background takes an indefinite cloudy or hazy stain which shows practically no detailed structures of its own and obscures the clear view of the contained fibrous neuroglia. In phosphotungstic acid hematoxylin preparations this background is a pale tan color not unlike that which the bodies of neuroglia cells take. One of Cajal's methods (given below) stains the fibrous neuroglia with startling clearness and at the same time stains in the cortex a tenuous interlacing meshwork which is apparently made up of extensions of the glia cell cytoplasm, and it seems probable that diffuse staining of this material is the reason for the lack of clearness by other methods.

A. Mallory's Phosphotungstic Acid Hematoxylin Stain.—

1. Fixation in Zenker's fluid, 24 hours.

2. Running water, 24 hours.

3. Dehydrate in graded alcohols (50 per cent., 60 per cent., 70 per cent. and 80 per cent.) each, 24 hours. Material may be left in 80 per cent. indefinitely.

4. Paraffin or celloidin embedding.

5. Treat sections with iodine solution (Gram's iodine solution or a 0.5 per cent. alcoholic solution) to remove the mercury precipitate, five to ten minutes.

6. Alcohol, 95 per cent., several changes to remove iodine.

7. Distilled water.

8. Permanganate of potassium 0.25 per cent. aqueous solution, for three to five minutes, sometimes ten to twenty minutes.

9. Wash in tap water.

10. Oxalic acid, 5 per cent. aqueous solution, five to ten minutes, sometimes longer.

11. Wash thoroughly in several changes of water.

12. Stain in phosphotungstic acid hematoxylin for twelve to twenty-four hours.

13. Transfer directly to 95 per cent. alcohol, followed by absolute alcohol for paraffin sections,and dehydrate quickly.

14. Clear in xylol (filter-paper blotting method for celloidin sections) and mount in xylol balsam.

Neuroglia, fibroglia, and myoglia fibrils and fibrin blue, collagen fibrils reddish-brown; the coarse elastic fibrils some times stain a purplish tint.

If after step 12 the sections are placed in a strong alcoholic solution (10 to 20 per cent.) of chlorid of iron for one to several minutes, followed by thorough washing in water, the collagen fibrils and other reddish-stained structures are completely decolorized.

Xylol must be used as the clearing reagent, because after origanum and other oils the blue color fades.

Sections stained in phosphotungstic acid hematoxylin keep for years if not unduly exposed to light.

This same staining method can be used after formaldehyde fixation if the tissues are first carried through Zenker's fluid in the ordinary way, but the results on the finer neuroglia fibrils are not so good.

B. Weigert's Differential Stain for Glia Fibers.—1. Fix thin pieces not over $\frac{1}{2}$ cm. thick in formalin for 24 hours.

2. Transfer to Weigert's glia mordant (page 143) for four or five days at room temperature. Steps 1 and 2 may be combined by adding 1 part commercial formalin to the 9 parts of glia mordant for the first 24 hours and then transferring to glia mordant without formalin for eight days.

3. Wash in water, dehydrate in alcohol, embed in celloidin.

4. Place sections for 10 minutes in 0.3 per cent. aqueous solution of potassium permanganate.

5. Rinse twice in distilled water and put into the following reducing fluid for 2 to 4 hours:

Chromogen	5 gms.;
Formic acid (Sp. Gr. 120)	5 c.c.;
Water	to 100 "

To each 90 c.c. of this add 10 c.c. of a 10 per cent. solution of sodium sulphite just before using, and filter carefully. The sections bleach rapidly but should be left in the solution as directed.

6. Rinse twice in distilled water.

7. Stain for a moment or two (the staining is practically instantaneous) in the following mixture:

Saturated solution of methyl violet in 70 to 80 per cent. alcohol (saturated with heat and decanted when cold),   100 c.c.;
5 per cent. aqueous solution of oxalic acid,   5   "

Staining is best accomplished on the slide.

8. Rinse and pour over the section a saturated solution of iodine in a 5 per cent. aqueous solution of potassium iodide. (Saturation with iodine is important.)

9. Rinse and decolorize thoroughly in a mixture of equal parts of xylol and aniline oil.

10. Repeated washings in xylol to remove all aniline oil. Xylol balsam.

Between steps 6 and 7 the sections may be left over night in a filtered 5 per cent. aqueous solution of chromogen, then rinsed and carried through as above. This affords the so-called contrast which gives the neuroglia fiber a somewhat darker color and stains the background pale yellow. Connective tissue stains blue after this step, however.

After staining, sections should be left in strong light for several days as this aids in fixing the stain.

C. *Bailey's Stain for Neuroglia Fibrils.*[1]—*Preparation of Dye.* To a saturated aqueous solution of ethyl violet diluted with three volumes of distilled water add a saturated aqueous solution of orange G drop by drop with a pipette, testing from time to time for the end point. This is done by letting a drop fall on filter paper. The ring which forms around the precipi-

[1] Bailey, Percival, J. Med. Research, 1923, XLIV, 73.

tate will be blue until the end point is reached, when it will become orange. Care should be exercised to stop just short of this moment.

The precipitated dye is filtered and the precipitate washed once with distilled water. After it has dried, dissolve it in absolute alcohol to form the stock solution.

Staining.—1. Zenker fixation, paraffin embedding and cutting.

2. Remove paraffin and pass to water through alcohol and iodin in the usual way.

3. Place for three days in a 3 per cent. aqueous potassium bichromate solution.

4. Rinse and place for twelve hours or longer in a solution of neutral ethyl violet-orange G made by diluting a portion of the stock solution with three times as much 20 per cent. alcohol.

5. Blot and agitate quickly in anhydrous acetone.

6. Place in toluol for a few seconds; then wipe most of it off the slide with a cloth.

7. Flood slide with pure clove oil.

8. Differentiate in clove oil 3 parts, 95 per cent. alcohol 1 part, agitating the slide and watching under the microscope till the small blood vessels show up as orange rings.

9. Blot and rinse in pure clove oil.

10. Blot and agitate in toluol; then xylol half a dozen changes.

11. Mount in balsam.

Neuroglia fibrils appear dark violet on an orange background. Nuclei, fibrin and red blood corpuscles are stained at the same time. The success of the stain depends on the freshness of the material. The best results are obtained after fixation in Zenker's fluid, but formalin-fixed tissue may be used after mordanting in the block or on the slide in Zenker's fluid or preferably in Bouin's fluid over night and then passing through the bichromate solution in the usual way.

The necessary dyes can be obtained from the National Aniline and Chemical Co.

The method, like other neuroglia procedures, stains also fibroglia and myoglia fibrils.

D. Cajal's Method for the Neuroglia.—1. Fix in the following solution:

Formalin,	14 c.c.;
Ammonium bromide,	2 "
Distilled water,	to 100 "

2. Frozen sections, 10 to 15μ.

3. Wash twice quickly, in water.

4. Place in the following staining fluid for 6 to 10 hours in the dark:

1 per cent. aqueous solution of gold chlorid,	10 c.c.;
5 per cent. aqueous solution of mercuric chlorid,	10 "
Water,	100 "

5. Wash 3 to 4 times in large amounts of distilled water.

6. Transfer for 15 minutes to the following fixing bath:

Sodium thiosulphate (hypo),	6 gms;
Potassium aluminum sulphate (alum),	1 "
Distilled water,	100 c.c.

7. Absolute alcohol. Xylol. Xylol balsam.

This method works to advantage only on fresh tissue and small pieces should be fixed. In successful preparations the ganglion cell bodies and vessel walls are faint purple or lavender while the glia structures are deep purple to black. This applies not only to the glia fibers but to the glia cell cytoplasm and to apparent fibril-like extensions of the glia cell cytoplasm in the gray matter where only a few differentiated glia fibers can be demonstrated and where many of the other glia methods give a mussy or clouded background.

Fieandt has published a method for staining both fibrous and plasmatic glia which is an application of Mallory's phosphotungstic acid hematoxylin to material fixed in Heidenhain's fixative.

2. **Stains for the Plasmatic Neuroglia.**—The chief methods for the neuroglia fibers (Mallory and Weigert) stain the latter selectively and leave the glia cell cytoplasm practically unstained. In early gliosis where marked degeneration of nerve

tissue is taking place and the glia cells are proliferating rapidly
to fill the defect but are not as yet actively producing fibers
the cytoplasm increases very markedly from the normal small
angular cell body to a large ovoid or irregular mass ("cellular
gliosis"). Often these big cells are crowded irregularly into
the interstices between white fibers and form relatively enor-
mous masses with multiple nuclei and no clear evidence of
division of their bodies. . These are the ameboid glia cells of
Alzheimer. They are short-lived structures occurring early
in the course of degenerative lesions. Some of them probably
go on to the production of fibers and thus take part in the scar
formation resulting in the fibrous glial replacement which is the
common end result of such lesions. Many, however, undergo
degeneration by fragmentation of their cytoplasm, resulting
in the formation of a mosaic of small angular masses staining
with variable intensity, the "Füllkörperchen" of Alzheimer.
The formation of amoeboid glia cells is apparently an acute
reaction and seems particularly profuse in rapid degenerations
of considerable masses of fibers and sheaths as, for example, in
subacute posterio-lateral sclerosis and much less frequently
in the slowly progressive processes like tabes. In chronic
meningitis (e.g., simple syphilitic infiltrative meningitis) there
is often a distinct zone formed between the marginal glia and
the outermost fibers of the cord made up of closely packed
ameboid cells. This appears at times as a protective walling
off of the cord tissues proper as there is frequently no demon-
strable loss of fibers underlying this mantle.

The same methods which show these cells also stain the cell
bodies of the neurophages in the same manner and may be used
for studies of this process.

The general stains such as the eosin-methylene blue will
frequently give a fairly good clue to the presence of the ame-
boid cells in numbers and good pictures are sometimes obtained
in hematoxylin Van Gieson and in toluidin blue preparations.
Three special methods are given here, however, which are
recommended for more accurate studies. All of these methods
are credited to Alzheimer.

A. Alzheimer's Modification of Mallory's Phosphomolybdic Acid Hematoxylin (Method IV).—1. Fix in Weigert's glia mordant direct for 2 weeks or in glia mordant plus formalin for 24 hours followed by glia mordant alone for two weeks. The former method is preferable.

2. Wash out in running water for 2 to 24 hours to remove the excess copper which otherwise will rapidly injure the knife edge. Prolonged washing seems to have no deleterious effect on the stain.

3. Frozen sections not over 10 to 15µ thick.

4. Wash two minutes in water to which a little acetic acid has been added (about 1 drop to 10 c.c.).

5. Carry over directly to a very weak solution of Mallory's phosphomolybdic acid hematoxylin (old formula) for 2 minutes.

10 per cent. aqueous solution of phospho-	
molybdic acid,	10 c.c.;
Hematoxylin,	1.75 gms.;
Carbolic acid,	5 "
Water,	200 c.c.

The solution must ripen for 6 to 8 weeks in a cotton-stoppered bottle exposed to the light. For the staining this solution is added drop by drop to a staining dish of distilled water until the mixture has just lost its transparency. Over ripe solutions are not so selective in their action.

6. Rinse and dehydrate rapidly in graded alcohols. Clear in xylol, xylol balsam.

The section should be reddish blue. Blue sections show a less selective staining.

The glia cell and nerve cell bodies are stained a pale bluish red, the axis cylinders in the white matter are darker. Ameboid glia cells vary considerably, even adjacent cells, in the intensity with which they are colored and often show deeply stained granular inclusions. Glia fibers are darker than the cytoplasm and well differentiated.

B. Alzheimer's Modification of Mann's Eosin-Methyl Blue Stain (Method V).—Steps 1, 2 and 3 as in preceding method.

4. Mordant 2 to 12 hours in a saturated aqueous solution of phosphomolybdic acid.

5. Wash quickly in two changes of distilled water.

6. Stain in Mann's solution 1 to 5 hours:

1 per cent. aqueous methyl blue solution,	35 c.c;
1 per cent. aqueous eosin solution,	35 "
Distilled water,	100 "

7. Wash in distilled water until color clouds are no longer given off.

8. 96 per cent. alcohol until the gray matter becomes light blue. The white matter should have a bright red or pink color.

9. Pass through absolute alcohol quickly; xylol, xylol balsam.

This method gives beautiful preparations of the white matter, and they keep well. Normal medullary sheaths are colorless to light pink depending on the degree of decolorization in the alcohol. Degenerating myelin sheaths are deep pink to red. Normal axis cylinders are deep blue or purple. Degenerating axis cylinders are glistening red. Glia cytoplasm is pale blue and the fibers dark blue. Ameboid glia cells vary from pale to dark blue as do the Füllkörperchen. Connective tissue is dark blue. In the gray matter the preparations are less acceptable. The nerve cells stain dark blue and show relatively little detail and the background also lacks clear cut differentiation. The method is particularly useful in demonstrating the ameboid neuroglia in the white matter and bids fair to become of service as a method for the early recognition of degenerating axis cylinders. The change from blue to red staining takes place in experimental material as early as 48 hours after a lesion while the other method by which this degeneration is usually demonstrated (the Marchi) depends on slow secondary changes in the medullary sheath, which do not begin to appear for about 7 days.

C. *Alzheimer's Light Green-Acid Fuchsin Method (Method VI).*

1. Fix in formalin 24 hours (not over 4 days at most).

2. Wash 24 hours in running water.

3. Transfer very thin pieces to Flemming's solution for 8 days in the dark. The solution should be in considerable volume in proportion to the tissue and should be changed once or twice during the period if it blackens.

4. Embed in paraffin and cut very thin sections, 2 to 4μ.

5. Stain in a saturated aqueous solution of acid fuchsin for 1 hour at about 60° C.

6. Let cool and wash in tap water.

7. Immerse in:

Saturated alcoholic solution of picric acid,   30 c.c.;
Distilled water,   60 "

This varies with the tissue from a single immersion to 2 minutes.

8. Wash in two changes of tap water.

9. Stain for 30 minutes to 1 hour in a 10 per cent. aqueous solution of light green.

10. Wash in two changes of tap water.

11. Differentiate to a violet color in 95 per cent. alcohol.

12. Absolute alcohol quickly. Xylol, xylol balsam.

The method gives extremely delicate pictures. Ameboid glia cells are varying shades of green and often contain fuchsinophile granules or brown-stained lipoid inclusions. The lipoid contents of perivascular phagocytes are brown or black. Glia fibers and red blood corpuscles are red, medullary sheaths unstained, connective tissues deep green, nerve cells pale green with red stippling, nerve cell nuclei darker green with a bright red nucleolus. The procedure is somewhat uncertain and must be varied with the tissue. The material must be fresh and pieces must be small. The color of the finished specimen to the naked eye should be neither red nor green but a delicate lilac. It often proves necessary to run several experimental sections in order to determine the optimum time in picric acid and light green but good preparations will repay this labor.

V. **Stains for Degeneration Products.**—The majority of the products of degeneration in the nervous system are of fatty nature and the most important methods for their demonstra-

tion are the scarlet red and osmic acid procedures. There are, however, a considerable number of other degenerative products.

The ordinary method of application of scarlet red in 70 per cent. alcohol as used for trunk tissues does not give the best results in the brain because of a tendency of the solution to tint the normal medullary sheaths. In its place is used the Herxheimer alkaline solution. The method is given here as it was in use in Alzheimer's laboratory.

A. **Herxheimer's Scarlet Red Stain.**—1. Fix in formalin.

2. Frozen sections 10 to 20μ.

3. Stain in the following solution:

Absolute alcohol,	70 c.c.;
10 per cent. aqueous solution of sodium hydrate,	20 "
Distilled water,	10 "
Scarlet red,	in excess.

This solution must be fresh and is ready with frequent shaking within 2 to 3 hours after mixing. After 36 hours it is no longer useful. The solution is carefully filtered into a deep watch glass (or small evaporating dish) covered with a second watch glass and heated until the upper glass sweats and then left for 15 to 20 minutes.

4. Rinse in distilled water.

5. Counterstain in diluted Ehrlich's hematoxylin (Delafield's does equally well), about 10 drops to 2 c.c. of distilled water for 10 to 20 minutes. The dilution should be of a weak port wine color.

6. Wash in tap water.

7. Mount in water and examine immediately or make permanent mounts in Kaiser's glycerine jelly (page 89).

Scarlet red stains not only the neutral fats and fatty acids produced by degeneration and autolysis of nerve cells but also many lipoid pigments.

B. **Marchi Method for Fatty Degeneration.**—This method is the classical one for the demonstration of secondary degeneration of the myelin sheath following death of the axis cylinder,

but can also be used to demonstrate fatty changes within the nerve cells, etc. In contrast with the Weigert stains, it gives a positive picture of the sheaths that are actually undergoing degeneration and has been the chief method for following the course of individual fiber groups and tracts. It is applicable, however, only to medullated fibers and the results of a lesion are not demonstrable for about a week after its occurrence. Normal sheaths reduce osmic acid to metallic osmium but previous treatment with a chrome salt so alters them that this reduction does not take place. The neutral fats, fatty acids and most lipochromes are not so altered, however, and hence blacken on exposure to osmic acid. Osmium which has been reduced by fat is soluble in ether, turpentine and xylol but much less so in alcohol, chloroform, oil of cloves and oil of origanum.

1. Fix in Müller's fluid—for fiber tract work preliminary hardening in formalin is possible but is not recommended. For the finer stippling of early fatty degeneration of the cells fixation should always be in Müller's fluid direct. Small pieces should be cut and fixed in Müller's fluid for 8 days to 3 weeks with frequent changes.

2. Transfer directly to the following solution for five to eight days in the dark with changes of the fluid when it blackens:

Müller's fluid,	2 parts;
1 per cent. aqueous solution of osmic acid,	1 part.

3. Wash overnight in running water.

4. Dehydrate and embed rapidly, in paraffin for cellular work or in celloidin for tract preparations. The steps of the embedding methods should be shortened as much as is consistent with proper impregnation. With small, thin pieces paraffin embedding (by the chloroform, not the xylol, method) may be accomplished satisfactorily in 12 to 16 hours.

5. Cut paraffin sections as thin as possible (4 to 6μ) and counterstain lightly, if desired, with carbol-fuchsin, or van Gieson's stain. Celloidin preparations for tract studies may be much thicker (20μ).

6. Clear in chloroform and mount in chloroform balsam (page 89).

Very thin pieces fixed for 24 hours in formalin, then washed out and left for one to two weeks in the dark in Flemming's fluid with changes as the fluid blackens, give excellent pictures of the fats in phagocytes and in the vascular endothelium but they are by no means so accurate as those by the Marchi method.

Some of the pigments contained in nerve cells are stained by both the scarlet red and osmic acid methods but resist solution in the embedding reagents and remain in alcohol fixed tissues, for example, as pale yellowish globular accumulations in the infranuclear zone of the cytoplasm. Some of these are probably extrinsic pigments derived from food stuffs and belong to the carotinoids. These may be stained more or less selectively according to Dolley either by overstaining in saturated aqueous solution of Nile blue sulphate and differentiating in weak acetic acid or as a progressive stain in a 1 : 10,000 aqueous solution acting for about half an hour. Others, particularly the relatively insoluble ones, are probably intrinsic.

Alzheimer has given several methods for the demonstration of various granules produced by degenerations of the glia and nerve cells. Among the most interesting of this group are the fuchsinophile granules which are brought out by the light green-acid fuchsin stain. Alzheimer considers these as precursors of the fatty degeneration products. These may be more strikingly stained by the following procedure:

1. Fix, mordant, embed and cut as for the light green-acid fuchsin method.

2. Mordant sections 1 hour at $37°$ C. in a saturated aqueous solution of copper acetate.

3. Wash twice in water and stain in the following solution for $\frac{1}{2}$ hour:

10 per cent. alcoholic solution of hematoxylin,	10 c.c.;
Distilled water,	87 "
Saturated aqueous solution of lithium carbonate,	3 "

4. Rinse in water, alcohol, xylol, xylol balsam.

The granules are stained blue-black on a pale background in which sufficient detail shows for orientation.

The methods in use for the demonstration of colloid droplets, hyalin, glycogen, calcification, etc., in the trunk organs can be applied with no modification to the nervous tissues.

METHODS OF FIXING AND EXAMINING SPECIAL ORGANS AND TISSUES

Tissues which are to be hardened should be obtained as fresh as possible. For this reason autopsies rarely furnish such perfect material as is obtainable from experimental lesions in animals or from surgical operations. Still, most of the pathological material comes from autopsies, and it is encouraging to know that very good work can often be done with tissues not fixed until twenty-four hours or even more after death. The most valuable autopsies are those which are freshest, and in which but one etiological factor has been concerned, so that the relation between the cause and the lesion produced is uncomplicated and can be readily grasped and understood.

The choice of the proper fixing reagent varies with the tissue, the lesion, and the use to which the material is to be put. It is advised as a routine procedure to preserve tissues in two fluids: in Zenker's fluid for general histological study, including both the injurious agents of all sorts and the inflammatory reactions to them; in formaldehyde for the preservation of fat and myelin, and certain substances to which it may be desirable to apply chemical tests. With these two fixatives properly applied it is possible to go a long way in pathological histology. Orth's fluid may be substituted for Zenker's fluid, but is distinctly not so good. Alcohol is required for the preservation of certain substances, such as glycogen, urate of sodium crystals, and hemosiderin, and corrosive sublimate fixation is necessary for certain special stains for mucin.

It is imperative that pieces of tissue for histological study should be placed in the proper fixative as soon after the removal

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of the organs from the body as possible, so that the surface will not dry or the blood and other fluids escape from the vessels. Do not wash off the surface with water. The tissues should almost invariably be cut into thin slices, not over 2 to 4 mm. thick.

In preserving tissues it is very important to use enough of the fixing reagent—ten to fifteen times as much as there is tissue. It is advised to harden tissues in flat-bottomed glass dishes and to stir them occasionally, so that they may come in contact with fresh fluid.

After Zenker fixation the best stain to use for general histological study is methylene-blue and eosin. For class use alum hematoxylin and eosin make a fairly satisfactory substitute, but do not demonstrate any bacteria present. The other useful stains are phosphotungstic acid hematoxylin, the aniline blue method for collagen fibrils, and Verhoeff's elastic tissue stain. After formaldehyde fixation the most interesting results are obtained by staining frozen sections with scarlet red and alum hematoxylin.

These methods of fixation and staining are applicable to most of the tissues listed below, and constitute the routine stains for almost all organs outside of the central nervous system, and even there they are often useful.

Acute Inflammatory Exudations; Granulation=tissue.—The elements in acute inflammatory exudations which require preservation are chiefly leucocytes of different sorts, serum, fibrin, and red blood-corpuscles. The best general fixative for them all is Zenker's fluid. It not only preserves perfectly the characteristic nuclei of the leucocytes, but also the cytoplasm, which stands out sharply in contrast-staining with eosin. The albumin of the serum is coagulated into a finely granular material. The fibrin and red blood-corpuscles stain brilliantly with eosin.

Lung.—In the preservation of the lungs it is important to save portions that have not been squeezed, so that the relations of the exudations may not have been changed or the alveoli compressed. Thin slices are usually preferable to cubical pieces, and should be cut with a very sharp knife, so as not to

compress the tissue, and dropped immediately into the fixing fluid, before the contents of the bronchi and of small cavities have had time to run out. An emphysematous lung is so delicate that it is usually better to inject a whole lung through the bronchi with the fixing fluid or to snip out small pieces with scissors. Zenker's fluid and formaldehyde are the most useful fixatives.

Spleen.—Owing to the presence usually of a large amount of blood in the tissue sections of the spleen must be cut very thin, preferably not over 2 to 3 mm. thick because the blood prevents the deep penetration of the fixing fluid. If the spleen is very soft it is sometimes advisable to cut the sections parallel with the surface so as to include the capsule which tends to hold things together.

Zenker's fluid is an excellent fixative for routine stains but formaldehyde is preferable for certain purposes such as the amyloid reaction and for the preservation of fat as in the endothelial leucocytes which are sometimes present in great numbers when lipemia complicates diabetes.

Bone=Marrow.—The pieces of tissue to be fixed should be about 2 mm. thick. The sections are to be cut in paraffin and are to be as thin as possible. For general purposes, fixation in Zenker's fluid and staining by the eosin-methylene-blue method are recommended. Other recommendations are:

1. For the study of erythroblasts and the formation of red blood-corpuscles: fixation in corrosive sublimate and staining by eosin soluble in alcohol and alkaline methylene-blue.

2. For the demonstration of the granules of myelocytes and leucocytes: fixation in corrosive sublimate and staining by Wright's blood-staining fluid undiluted, then washing in water, dehydrating in acetone, clearing in oil of turpentine, and mounting in turpentine colophonium. The Biondi-Heidenhain triple stain may also be used.

Schridde's Method of Staining the Granulations of Myelo= cytes and Leucocytes in Sections.—The tissue is best fixed in Orth's fixing fluid, but other fixatives may be used. The sections should not be thicker than 5μ and should be fixed

to the slide with Mayer's albumen mixture. They are stained for twenty minutes in Giemsa's stain, diluted with distilled water in the proportion of two drops of the stain to each cubic centimeter of water. The mixture must be freshly made before using. When the staining is completed the preparation is washed in water, the excess of water removed with filter-paper, and the section immediately placed in pure acetone. If the acetone extracts color from the preparation, it is impure and should not be used. The section is then cleared with toluol or xylol and embedded in neutral xylol balsam. Care should be taken not to allow the sections to become dry from the rapid evaporation of the acetone.

The neutrophile granulations are stained a violet-red; the eosin granulations' red; the granulations of the mast-cells dark blue, and the granulations in the cytoplasm of the megakaryo-cytes violet-red. All nuclei are blue and the red blood-corpus-cles grass-green. The connective tissue is of a pale red color.

Wright's Method for the Differential Staining of the Blood-platelets and the Giant Cells (Megakaryocytes) of the Bone-marrow.—The tissue should be absolutely fresh. It is fixed in a 4 per cent. aqueous solution of formaldehyde or in a satu-rated solution of corrosive sublimate in 0.9 per cent. salt solution. Tissue that has been decalcified is not suitable. The sections are cut in paraffin and should not be more than 7 microns thick. They are stained while affixed to the slide by Mayer's egg-albumen method.

The staining fluid and the mode of its preparation are described below.

The staining, clearing, and mounting are carried out as follows:

1. Equal parts of the staining fluid and distilled water are mixed in a small wineglass and immediately poured on to the slide. The measuring is conveniently done by means of a small pipette provided with a rubber bulb. At least 2 c.c. of the freshly diluted staining fluid are thus spread out over the slide, which should be supported upon some object in such a way as to prevent the fluid from running off. The

spreading out of the fluid in a layer is important, because it facilitates the evaporation of the alcohol, whereby the staining elements slowly precipitate out of solution and, while doing so, stain the tissue elements. This precipitate appears as a yellowish, metallic scum which slowly forms on the surface of the mixture. The diluted staining fluid is allowed to act for about fifteen minutes, when the preparation is immediately washed in water. The exact time required for the best results has to be determined for each batch of the staining fluid. The proper staining of the preparation may be judged by examining it under a low magnifying power by artificial light after pouring back the diluted staining fluid into the wineglass. The staining is stopped, by washing the preparation in water, when the cytoplasm of the giant-cells has acquired a bright red color and the fibrils of the reticulum begin to take on a red color also. If the staining is found not sufficiently intense, the diluted staining fluid is poured back on the preparation and allowed to act longer. Overstaining and formation of a black-red granular precipitate on the preparation occur if the diluted staining fluid is allowed to act longer than a certain time.

2. Dehydrate in pure acetone.

On account of the great volatility of acetone, some care is necessary to prevent the drying of the preparation, which should be avoided.

3. Clear in xylol or pure oil of turpentine.

4. Mount in a thick solution of colophonium in xylol or pure oil of turpentine.

Before mounting the preparation the superfluous turpentine should be removed, because this reagent rapidly takes up water from the air, and thus may cause clouding of the preparation or fading of the stain.

The solution of colophonium is made by saturating a quantity of turpentine with powdered colophonium, and keeping the filtered solution in the paraffin embedding oven until it has evaporated to the required consistence.

The use of acetone instead of alcohol for dehydrating is an important feature of the method, for the latter spoils the

characteristic staining of the granules in the giant-cells and platelets.

The *staining fluid* is composed of 1 part of a modified methylene-blue solution and 10 parts of a 0.1 per cent. solution of water-soluble eosin in pure methyl alcohol.

The solution of methylene-blue is prepared as follows: One gram of methylene-blue "med. pur." is dissolved as thoroughly as possible in 100 c.c. of an 0.5 per cent. aqueous solution of sodium bicarbonate in an Erlenmeyer flask. The flask and its contents are then placed in an ordinary steam sterilizer and kept at 100° C. for one hour and a half, counting the time after the steaming has become vigorous. When cool, the mixture is filtered and the filtrate is the modified blue solution. It must be of a well-marked purple color when viewed in a thin layer by the yellow transmitted light of an ordinary incandescent electric bulb. This color appears only after cooling.

Variations in the solutions of the blue and of the eosin may require that the proportions above given be changed slightly. An excess of eosin delays the appearance of the scum on the surface of the diluted staining fluid, and prolongs the time required for staining. On the other hand, an excess of the modified blue component hastens the appearance of the scum, and may cause overstaining and the granular precipitate to form on the preparation.

The blood-platelets typically appear as rounded bodies, more or less jagged in outline, and composed of a hyaline, blue-staining substance, in which are embedded, chiefly in the central portions, fine red to purplish granules. The cytoplasm of the giant-cells shows the same structure and staining peculiarities. The sections should be examined by an incandescent electric light in order to bring out the colors to the best advantage. By this method all grades of transition can be shown between pseudopod-like processes of the giant-cells or detached masses of giant-cell cytoplasm and blood-platelets.

Smear preparations may be made either upon slides or cover-glasses, and stained by Wright's blood-stain, as in the case of blood-smears. For the best results the preparation

should not be allowed to dry, but should be stained immediately while still wet, and, after staining, dehydrated with absolute alcohol, cleared with xylol, and mounted in balsam in the same manner as a section affixed to the slide. A longer period of staining than that directed for blood-smears is usually desirable.

Special Methods for Smear Preparations from Bone-marrow.

Thoroughly tease a small bit of the marrow in a few drops of blood-serum and from this mixture prepare the smear preparation, as in the case of a blood-smear. The preparation, however, must not be allowed to dry, but is fixed immediately by methyl-alcohol (one minute) *while still wet*. It is then covered for three to five minutes with a mixture of equal parts of Wright's blood-stain and distilled water. This mixture must have been prepared immediately before use. After staining, the preparation is not allowed to dry, but is washed in water, dehydrated with acetone, cleared with oil of turpentine, and mounted in turpentine colophonium. Instead of methyl-alcohol, corrosive sublimate, Zenker's fluid, or a 1 per cent. solution of osmic acid may be used for fixation, each being allowed to act about one minute, when the preparation is to be washed in water, covered with 95 per cent. alcohol for one minute, rinsed in water, and then treated as indicated. It is important that the preparation be not allowed to dry at any stage of the process.

By these methods many of the finer details of the marrow-cells are brought out much better than by the usual smear method.

Kidney.—Zenker's fluid and formaldehyde as fixatives answer most purposes, but alcohol is required to preserve glycogen and certain crystalline deposits. The scarlet red stain, after formaldehyde fixation, has to a large extent replaced Flemming's solution. Fixation by boiling is still used to demonstrate an albuminous exudate in the capsular space. The general staining methods already recommended will be found the most satisfactory.

In cases of chronic nephritis the capsule should not be peeled from those parts kept for microscopical purposes.

Paraffin embedding is generally to be preferred for the kidney, especially when lesions of the glomeruli are present.

Gastro=intestinal Tract.—Portions of the stomach or intestine should be hardened as soon after death as possible for satisfactory study, because the gastro-intestinal tract so rapidly undergoes post-mortem changes. It has been recommended in appropriate cases, where an autopsy is allowable, to inject the stomach with the desired fixing solution by means of a rubber tube as soon after death as is permissible. Under no circumstances should the surface of the intestine or stomach be washed with water. Use either normal salt solution or some of the fixing solution. It is important to keep the tissue flat while hardening. This can usually be done by laying it with the peritoneal surface down on thick filter-paper, to which it readily sticks. Sometimes it is necessary to pin the specimens down at the edges on flat pieces of cork. Do not let the surface dry before the specimen is placed in the fixing solution. Zenker's fluid can be highly recommended as a fixative, but alcohol is sometimes to be preferred.

Liver.—Fat is most easily and satisfactorily demonstrated by the scarlet red stain after formaldehyde fixation. Necrosis of liver-cells is best shown by the eosin-methylene-blue stain after fixation in Zenker's fluid. The necrotic cells stand out of a deep pink color in sharp contrast to the other cells. The aniline blue stain is especially useful in the study of the lesions associated with chronic passive congestion and with amyloid deposit.

For obtaining the iron reaction with hemosiderin in cases of pernicious anemia and hemochromatosis and for the reactions of amyloid, harden in alcohol or formaldehyde.

For general histological study Zenker's fluid will be found exceedingly useful. The iron reaction can be obtained after it by the ammonium sulphide and ferricyanide of potassium method.

The bile-capillaries may be demonstrated by the same method that is used for neuroglia fibrils—namely, fixation in Zenker's fluid, following by staining in phosphotungstic-acid hematoxy-

lin. The treatment with permanganate of potassium and oxalic acid must be more prolonged than usual, however, otherwise the albuminous granules in the cytoplasm will stain too deeply and obscure the capillaries.

For Eppinger's elaborate method, see *Ziegler's Beiträge*, vol. xxxi.

Pancreas.—Much interesting and valuable work has been done recently on the histology of the pancreas, especially with reference to the cytoplasmic granules in the different kinds of cells of the ducts, glands, and islets. Some attempt has been made to apply the methods to the lesions of the pancreas, more particularly to those associated with the syndrome known as diabetes mellitus. The results so far obtained are promising and encourage further study along the same lines.

For routine microscopic study of the pancreas, fixation in Zenker's fluid and staining by the eosin-methylene-blue method are recommended. The zymogen granules do not stain intensely, as in the glands of the stomach and intestine after this procedure, but require special methods to render them prominent. Staining with phosphotungstic acid hematoxylin after fixation in formaldehyde is sometimes useful. Fixation in formaldehyde is also advisable for certain other purposes, such as the examination for fat, hemosiderin, and amyloid.

As the best method for staining all the various granules in the cells of the pancreas, Bensley recommends that used for mitochondria (see page 110).

For the specific granules of the A and B cells in the islets, the best technique in Bensley's opinion is the neutral gentian stain after fixation in chrome sublimate.[1]

A. Fix in the following solution:

Potassium bichromate,	2.5 gms.;
Mercuric chlorid,	5.0 "
Distilled water,	100.0 "

[1] For other methods of fixing and staining the islet cells consult these papers: Bensley, R. R., "Studies on the Pancreas of the Guinea Pig," *Amer. Jour. of Anat.*, 297–388, xii, 1911; Lane, M. A., "The Cytological Characters of the Areas of Langerhans," *Amer. Jour. of Anat.*, vii, 1907.

It is Zenker's fluid minus the acetic acid, as the latter dissolves both mitochondria and the characteristic granules of the islet cells.

The stain used is Bensley's neutral gentian, the name given to the neutral dye obtained when a solution of gentian violet is precipitated by its equivalent of a solution of Orange G. The dye is prepared as follows:

A. Gentian violet, 1 gram in 25 c.c. of water.

B. Orange G., 1 gram in 25 c.c. of water.

Add *A* to *B*, shaking gently, until practically complete precipitation has taken place. Filter and wash with water at once. Drain and dry. Dissolve residue in 25 c.c. of absolute alcohol. For staining, add the stock solution of the neutral compound to 20 per cent. alcohol until a solution having the color of a good hemalum solution is obtained. Allow this solution to stand twenty-four hours to permit the excess of dye to separate out, when it may be employed for staining as follows:

1. Stain in neutral gentian violet solution twenty-four hours.
2. Blot between several layers of filter paper.
3. Dehydrate in acetone.
4. Place sections in toluol.
5. Differentiate in

Absolute alcohol,	1 part;
Oil of cloves,	3 parts.

6. Wash with toluol and mount in balsam.

In the stain for mitochondria the granules in the A cells are stained deeply red, those in the B cells green. After the neutral gentian violet stain, followed by staining in acid fuchsin, the granules of the A cells are stained red, those of the B cells, violet.

Goodpasture has found eosin and his acid polychrome methylene-blue solution to afford a very useful method for staining differentially the zymogen and the alpha and beta granules after fixation in neutral Helly's fluid or in neutral Orth's fluid.

1. Fix thin pieces of fresh pancreas for twenty-four hours in

Neutral formaldehyde,	10 c.c.;
Zenker's fluid without acetic acid,	90 "

Or in

Neutral formaldehyde	10 c.c.;
Bichromate of potassium (2.5 per cent.),	90 "

2. Wash in running water twenty-four hours.

3. Dehydrate in alcohol; embed in paraffin and cut sections; pass through xylol and alcohol to water in the usual way.

1. Potassium permanganate, 1 per cent. aqueous solution, one minute.

2. Oxalic acid, 5 per cent. solution, one minute.

3. Wash thoroughly in water.

4. Stain in aqueous solution containing 1 per cent. eosin and 1 per cent. bichromate of potassium for one to five minutes.

5. Wash hastily in water.

6. Acid polychrome methylene-blue, one to five minutes.

7. Wash hastily in water.

8. Differentiate and dehydrate rapidly in 95 per cent. and absolute alcohol.

9. Xylol and balsam.

In properly stained sections xymogen granules stain deep purple; cytoplasm, light blue; nuclei, light purple; alpha granules, brick red, and beta granules, dark blue.

Bone and Cartilage.—Excellent work can be done after hardening in alcohol, and fixation in it is generally recommended for all infectious processes in bone. The histological structure is, however, better preserved in Zenker's or Orth's fluid. In decalcifying bone, after proper fixation, thin pieces should be taken, not more than 2 to 4 mm. thick, so that the process may be finished as quickly as possible. While tubercle bacilli will stain readily after being twenty-four or even forty-eight hours in 5 per cent. nitric acid, it is impossible to stain them after they have been subjected to the same strength of nitric acid for four days. (For details in regard to decalcification see page 49.)

Celloidin is preferable to paraffin for embedding. Besides a simple stain with alum-hematoxylin, double stains of the latter with neutral carmine or eosin are sometimes advantageous. The best pictures with carmine as the contrast-stain are obtained by staining first in alum-hematoxylin, washing twelve to twenty-four hours, and then staining in the neutral carmine. The carmine stains decalcified bone and osteoid tissue red. Phosphotungstic-acid hematoxylin will sometimes be found useful, especially when cartilage is present, because it stains the intercellular substance, both of bone and of cartilage, pink, while the nuclei are stained blue. The ground substance of cartilage, especially in new-growths, often stains so intensely with alum-hematoxylin that the nuclei are quite obscured. For the same reason chlorid of iron hematoxylin is often useful because it does not stain the ground substance.

The following method is recommended for differentiating cartilage from bone:

Schaffer's Safranin Method.—Decalcify with nitric acid.

1. Stain sections a half to one hour in an aqueous solution of safranin, 1:2000.

2. Wash in water.

3. Place for two to three hours in a $\frac{1}{10}$ per cent. solution of corrosive sublimate.

4. Examine in glycerin, or, if permanent specimens are desired, pass very quickly through alcohol, blot with filter-paper, further dehydrate, and clear for a long time in bergamot or clove oil, and mount in xylol balsam. This is a double stain: cartilage, orange; bone, uncolored; connective tissue and marrow, red.

None of the methods above given has proved reliable in the study of rickets and of osteomalacia for differentiating osteoid from true bone-tissue. In important cases, therefore, it is advisable to use an old knife, and to cut sections of the undecified tissue after embedding thoroughly in celloidin.

Schmorl's methods of demonstrating the lacunæ and canaliculæ of bone in sections can be highly recommended.

Method A.—1. Fix, preferably in Müller's fluid, formaldehyde, or Orth's fluid; do not use corrosive sublimate solution.

2. Decalcify by the slower methods—namely, Ebner's or Thoma's, or in Müller's fluid 100 c.c. plus nitric acid 3 c.c.

3. Embed in celloidin; paraffin is objectionable.

4. Place the sections for at least ten minutes in water to get rid of the alcohol.

5. Stain for five to ten minutes or longer in saturated solution of thionin in 50 per cent. alcohol, 2 c.c., water, 10 c.c., or in Nicolle's carbolthionin solution:

> Saturated solution of thionin in 50 per cent.
>   alcohol,   10 c.c.;
>   1 per cent. carbolic acid water,   100 "

6. Wash in water.

7. Place in a saturated aqueous solution of picric acid for one-half to one minute.

8. Wash in water.

9. Place in 70 per cent. alcohol for about five to ten minutes until no more dense clouds of color are given off.

10. Dehydrate in 95 per cent. alcohol.

11. Clear in oleum origani cretici.

12. Xylol balsam.

Bone substance yellow to yellowish-brown; bone lacunæ and canaliculæ dark brown to black; cells red. Fat-cells after fixation in Müller's fluid reddish violet. Osseous tissue stains a deeper yellow than osteoid tissue. Canaliculæ stain in osseous tissue, but not in osteoid tissue unless the thionin solution is made alkaline by the addition of 1 or 2 drops of ammonia. (This solution cannot be recommended for general use.)

This method is not a true stain, but resembles Golgi's method; a precipitation of coloring-matter takes place in the lacunæ and canaliculæ; it also takes place to a considerable extent in other narrow spaces in the tissues, and often is very disturbing. It can be gotten rid of to some extent without injury to the stain by leaving the sections in step 8 in the water

for half an hour. The canaliculæ are now usually brownish red to red, and the bone substance blue to colorless. In this case it is often best to stain the sections first in alum hematoxylin to bring out the nuclei.

Method B gives good results with the bones of children only.

1. Harden in Müller's fluid or in Orth's fluid, followed by Müller's for six to eight weeks, or for three to four weeks in the thermostat; take very thin pieces of tissue.

2. Wash off in water, and decalcify in Ebner's solution.

3. Wash thoroughly in running water.

4. Harden in alcohol; embed in celloidin; cut sections very thin.

5. Stain in Nicolle's carbolthionin, or better in the alkaline (NH_4OH) thionin solution given above, for three minutes.

6. Transfer to a saturated aqueous solution of phospho-tungstic or phosphomolybdic acid (use glass or platinum needle) for a few seconds or longer. The sections become blue, green, or gray in color.

7. Water five to ten minutes until they acquire a sky-blue color.

8. Place in dilute ammonia (1–10) for three to five minutes to fix the color.

9. Transfer directly to 90 per cent. alcohol; change several times to get rid of the ammonia.

10. 96 per cent. alcohol.

11. Clear in carbol-xylol.

12. Xylol colophonium or balsam.

If the ground-substance is stained too deeply by the alkaline thionin solution, treat the sections with acid alcohol for five minutes, followed by water before dehydrating. The borders of the lacunæ and canaliculæ stain bluish black; the ground-substance of bone clear to greenish blue; cellular elements a diffuse blue color. In rachitic bones the canaliculæ are brought out only in osseous tissue.

Skin.—Much of the material for the study of lesions of the skin is obtained during life by means of the knife or scissors. Fixation in absolute alcohol is often advisable, especially when

it is desired to stain bacteria, mastzellen, plasma-cells, and elastic fibers. The staining methods for these tissue elements will be found on pages 112–115. For Unna's innumerable stains for degenerated connective-tissue fibers, elastic fibers, etc., the reader is referred to his numerous articles on technique in the *Monatsheft f. prakt. Dermatologie.*

For many skin-lesions, especially those in which blood-vessels play a more or less prominent part, Zenker's fluid is advisable. Clear tissues in oil of cedar wood rather than in chloroform for paraffin embedding as it does not render them quite so brittle as chloroform does.

In the examination of hairs or scales of epidermis for bacteria and fungi it is important first to remove the fat from them by means of equal parts of alcohol and ether. They are then examined in 40 per cent. caustic potash, which, by clearing up the cells, brings out the organisms and spores quite distinctly. Heating the potash over a small flame hastens the process, but is a somewhat risky proceeding; soaking in the solution over night is better. Examine the preparation with most of the light excluded.

Preparations may be made in certain cases by touching the cover-slip to the surface of the lesion, drying, and passing through the flame. After removing the fat by means of alcohol and ether, stain as with ordinary cover-slip preparations.

Unna's method is to rub up the scales of epidermis in a little glacial acetic acid between two slides, which are then drawn apart and quickly dried over the flame. After removing the fat by means of alcohol and ether the slide preparations are stained in borax-methylene-blue.

For staining the various vegetable parasites of the skin Malcolm Morris recommends the following method, which he claims is the best one yet devised, as it avoids the use of the hydrate of potash:

1. Ether or alcohol and ether equal parts.

2. Stain in a solution of 5 per cent. gentian-violet in 70 per cent. alcohol, five to thirty minutes.

3. Iodine solution, one minute.

4. Aniline, or aniline plus 2 to 4 drops of nitric acid.

5. Aniline.

6. Xylol.

7. Xylol balsam.

The most suitable medium for the growth of the various ringworms is the following, due to Sabouraud:

Agar-agar,	1.30
Peptones,	.50
Maltose,	3.80
Water,	100

Instead of test-tubes, Erlenmeyer flasks are used, so as to get a large flat surface for the growth to spread over from the point of inoculation in the center. The most favorable temperature for growth is 30° C.

MUSEUM PREPARATIONS.

Specimens intended to be preserved for the museum should generally be gotten into pretty good shape by trimming and dissecting before they are placed in the hardening reagent. Of the liver and other large organs and tumors, sections 1–3 cm. thick are generally to be preferred to the whole specimen. It is obvious that the center of any large organ must undergo marked post-mortem changes before any fixing agent we yet possess except heat can penetrate it beyond 1–2 cm. Injection through the blood vessels is of course possible and often practiced, especially with the brain, but is not aways applicable to other tissues.

When only form and external appearance require preservation the condition of the inside of the specimen may be neglected except that it must be acted on by the fixing fluid even if weeks to months are required before the specimen can be considered preserved. Alcohol and formaldehyde are often used for preserving gross specimens when only form and size enter into consideration. For colors they are practically useless.

After the introduction of formaldehyde as a fixing agent much was expected of it as a preservative of natural colors. Its

failure in this respect, however, was soon realized. In 1896 Melnikow-Raswedenkow published a method for redeveloping the colors of formaldehyde-fixed tissues by means of alcohol and their preservation in a solution of potassium acetate and glycerin in water. In the same year Kaiserling announced a greatly improved fixing solution made by adding potassium acetate and potassium nitrate to the formaline solution, and adopted the developing and preserving steps proposed by Melnikow-Raswedenkow. The combined method generally goes under his name.

There have been numerous modifications of Kaiserling's fixative but none has surpassed it and it is exceedingly doubtful if any has equalled it, at least in the preservation of the colors due to hemoglobin and its derivatives. It can be highly recommended. Trust reposed in some of the other fixatives, especially those containing a low percentage of formalin, has led to the ruin of many good pathological specimens particularly during the war-time when the cost of glycerin and other chemicals mounted high.

It is usual to develop the colors in Kaiserling preparations by using first 80 and then 95 per cent. alcohol. Some workers, however, advise the use of 95 per cent. alcohol only.

The preserving fluid like the fixative has been variously modified. It is usual to saturate it with thymol to prevent the growth of mold. Carbolic acid, much used at first, has not proved satisfactory. Recently saturation with trioxide of arsenic has been highly recommended for the permanent preservaton of the colors.

Pick's modification of Kaiserling's fixative, making use of Carlsbad salts instead of nitrate and acetate of potassium, was introduced in 1900 and has been much used and often modified. It was originally followed by the same method of development and preservation as was employed for Kaiserling specimens.

According to Puppe, oxyhemoglobin in formaldehyde changes to acid hematin, and then in alcohol to alkaline hematin which is red and so like the color of oxyhemoglobin that colors due to

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the presence of blood appear normal and the tissues natural.

For the best preservation of colors in Kaiserling and similar preparations fill the jars full, seal them air tight, and protect them from the light so far as possible.

With large organs or tumors weeks to months may be required to obtain complete fixation of blood stained fluid, and this must be done or it will later discolor the specimen.

Kaiserling's Method of Preserving the Natural Colors in Museum Preparations.—1. Fixation for one to five days in—

Formaldehyde,	200 c.c.;
Water,	1000 "
Nitrate of potassium,	15 gms.;
Acetate of potassium,	30 "

Change the position of the specimen frequently, using rubber gloves to protect the hands from the injurious effect of the formaldehyde. The time of fixation varies with the tissue or organ and size of the specimen.

2. Drain and place in 80 per cent. alcohol one to six hours, and then in 95 per cent. alcohol for one to two hours, to restore the color which is somewhat affected in the fixing solution.

3. Preserve in—

Acetate of potassium,	100 gms.;
Glycerin,	200 c.c.;
Water saturated with thymol,	1000 "

Exposure to light gradually affects the colors. The process of fixation should be performed in the dark, and the specimens when preserved should be kept in the dark except when on exhibition.

If it seems desirable to cut a thin slice from the face of a specimen, this should not be done until the preparation has been in the preservative fluid two weeks. The specimen may then be placed in alcohol for one to two hours to brighten up the colors.

Pick's method employing Carlsbad salts has been modified by adding chloral hydrate (Jores) and by increasing

the percentage of formaline (Silvester). Artificial Carlsbad salts according to the formula of Klotz and MacLachlan are generally substituted for the natural product.

Artificial Carlsbad Salts (Klotz and MacLachlan).

Sodium sulphate	22 gms.
Sodium bicarbonate	20 "
Sodium chloride	18 "
Potassium nitrate	38 "
Potassium sulphate	2 "

1. **Fixing Solution.**

Chloral hydrate,	50 gms.;
Carlsbad salts, artificial,	50 "
Formalin,	100 c.c.;
Water,	1000 "

The directions are the same as for Kaiserling's fluid. Thorough fixation is important.

2. **Washing.**—Development of colors in alcohol is not necessary in this modified method because owing to the presence of chloral hydrate they are not lost. On the other hand, thorough washing in running water, for 3–24 hours, is generally advised in order to get rid of all traces of chemicals but especially of the formaldehyde.

3. **Preserving Solution.**—The preserving fluid originally recommended by Melnikow-Raswedenkow has been modified by the addition of arsenious acid (Delépine, Silvester) which improves the color keeping properties of the solution. Satur-

Potassium acetate,	300 gms.;
Glycerin,	300 to 600 c.c.;
Saturated arsenious acid water,	1000 "

ated arsenious acid water is made by boiling an excess of trioxide of arsenic in water for two hours. Allow to cool and filter or decant off the clear fluid.

For display purposes the preparations preserved by these methods are often mounted permanently in gelatin. A jelly is made by adding ten parts by weight of gelatin to the third solution in which the specimens are ordinarily kept. The same procedure should be followed as in the making of Kaiser's glycerin jelly for the mounting of histological sections. For a

preservative add 1 per cent. of carbolic acid. Formaldehyde is often used for this purpose, but in spite of it, or owing to its evaporation, ferments present in the tissues (as shown by L. J. Rhea) often result in liquefaction of the jelly in the course of weeks to months.

Owing to the discoloration which sometimes takes place in the jelly and the difficulty of remounting the tissues, L. W. Williams advises using only a layer of the jelly sufficient to attach the specimen to the back of the jar, and filling up the space in front with the third solution, which can easily be renewed. By this method the specimen is held firmly in place and is viewed through a clear, colorless medium.

PATHOLOGICAL PRODUCTS

Cloudy Swelling; Albuminous Degeneration.—The increase in the relative number of the albuminous granules of the cytoplasm of the various tissue-cells in pathological processes is usually determined by examination of the fresh material, either macroscopically from the appearances on section, or microscopically from teased preparations or frozen sections mounted in salt solution. The organ as a whole (and therefore the individual cells) usually shows some increase in size. The nucleus is generally more or less obscured if the process is at all marked. According to Israel, the cloudiness must be recognizable with low powers and in places where the cells are massed together. The diagonsis should not be based on the appearances of single cells.

The chemical properties of the albuminous granules are the following: they disappear on treatment with dilute acetic acid (1–2 per cent. solution usually); they are not dissolved by chemical substances which dissolve fat (absolute alcohol, ether, chloroform, etc.); and they do not stain with osmic acid. The acetic-acid test is the one usually employed.

For demonstrating changes in size and number of the albuminous granules in sections of fixed tissue use the Altmann-Schridde method on page 112. The formation of hyaline droplets, which occurs in the late stage of the process, is best

shown by Pfister's modification of Weigert's fibrin stain (decolorization with a 1 to 4 aniline-xylol mixture). The droplets are stained deep blue.

Fats and Lipoids (Lipins).—Under this heading is included a large number of chemically related substances which are divisible into several groups. It is important to have some idea of their classification and relations because examples of many of them occur in the human body under normal and pathological conditions, rarely, however, in pure state, but usually in mixtures of various composition. These different lipins can be recognized positively only by chemical methods, but many of them possess more or less definite morphological or physical properties or staining reactions which distinguish them from other substances and to some extent from each other.

1. **Fats and Oils, Fatty Acids, Soaps.**—The true neutral fats and oils are esters of glycerol (an alcohol) with fatty acids (oleic, palmitic, stearic, etc.). They contain only C, H and O, no N or P. The fatty acids combine with Ca, Na and K to form soaps.

2. **Cholesterines.**—Lipoids of the aromatic series, containing only C, H and O, no N or P. They occur free or in combination with fatty acids as esters of cholesterol (an alcohol).

3. **Cerebrosides** (phrenosin, kerasin) contain C, H, O and N (fatty acids, nitrogen and a carbohydrate group) but no P.

4. **Phosphatides** (lecithin, kephalin, sphingomyelin) contain C, H, O, N and P (fatty acids, nitrogen and phosphorus).

5. **Waxes.**—Esters of sterols and fatty acids.

The more or less common properties on which the microscopic recognition of fats and lipoids is based are the following:

1. *Solubility* in alcohol, ether, chloroform, benzine, etc., *insolubility* in water, acids, alkalies.

2. *Refractiveness.*—In water small drops appear dark; large drops have dark contours and light centers; some lipoids are doubly refractive when viewed through Nicoll prisms.

3. *Reduction of osmic acid* (osmium tetraoxide) as a result of which they are stained black (applies chiefly to oleic acid and its compounds).

4. *Staining with certain aniline dyes* which are soluble in fats (applies to the fats but to only a part of the lipoids).

Three different types of stains are employed for demonstrating fats and some of the lipoids: osmic acid, scarlet red and Nile blue sulphate. Of these the most important and generally useful is scarlet red. In addition special staining methods have been devised for certain lipoids (medullary nerve sheath, acid-fast bacilli).

The methods of demonstrating true fats will be given first and then the application of these and other methods to the staining of some of the other lipins.

Examination for fat can be made in the fresh state either in teased preparations or in frozen sections; in smears; and after fixation.

For teased preparations and frozen sections of fresh tissue it is usual to employ acetic acid by drawing it beneath an edge of the cover-slip by means of filter paper placed at the opposite edge. The albuminous granules disappear; the fat droplets show up owing to their refractiveness. Scarlet red and osmic acid can be used to stain the fat but are best reserved for fixed tissues.

Smear preparations can be fixed in formaldehyde vapor for five to ten minutes and then stained by scarlet red or by exposure to the vapor of osmic acid. The staining methods for fat in tissues are best applied to frozen sections of material which has been fixed in formaldehyde.

Osmic Acid Stain for Fat.—Osmium tetraoxide (OsO_4) is reduced to osmium oxide (OsO_2) by oleic acid and its compounds (also by certain other substances such as tannic acid and eleidin) which are thereby stained black. Palmitic and stearic compounds are stained only when exposure to osmic acid is followed by treatment with alcohol which changes it to osmium hydroxide ($Os(OH)_4$), a process called secondary staining.

Fat can be stained by fixing tissues in solutions such as Flemming's or Marchi's which contain osmic acid, but there is always danger of some of the osmium reduced by fat being dissolved out in ether, turpentine, xylol and toluol. In embedding in paraffin avoid xylol; use chloroform instead; and mount in properly prepared chloroform balsam or colophonium. The

best contrast stain is saffranin as employed for mitotic figures but the step containing acid alcohol must be omitted. Embedding in celloidin is not contra-indicated, as the alcohol probably protects the osmium from the injurious action of the ether. For clearing use oleum origani cretici. A better method is to stain the fat in frozen sections of formaldehyde fixed tissues.

Directions.—1. Fix in 10 per cent. formalin for twenty-four hours.

2. Cut frozen sections.

3. Place in a 1 per cent. solution of osmic acid for twenty-four hours (Flemming's or Marchi's solution can be used instead).

4. Wash thoroughly in running water for six to twelve hours.

5. Place in absolute alcohol for several hours to obtain the secondary staining of fat not yet colored.

6. Wash in water.

7. Mount in glycerin jelly.

Sudan IV, Scarlet Red (Michaelis) Stain for Fat.—Sudan III (Daddi) was used originally but is less brilliant. The solution of scarlet red most commonly used has been a saturated one in 70 per cent. alcohol. It requires staining over night in order to obtain the best results. Recently, G. Herxheimer has advised a saturated solution in—

70 per cent. alcohol,	50 c.c.;
Pure acetone,	50 "

Staining takes place very quickly and is intense and sharp. The solution can be highly recommended and should take the place of any other yet proposed.

Cover-slip Preparations.—1. Fix in the vapor of formaldehyde for five to ten minutes.

2. Stain in the alcohol-acetone solution of scarlet red for two to five minutes.

3. Dip for an instant in 70 per cent. alcohol.

4. Wash in water.

5. Counterstain with alum hematoxylin or methylene-blue.

6. Wash in water.

7. Mount in glycerin or glycerin jelly.

Sections.—1. Make frozen sections of formaldehyde-fixed tissue.

2. Dip for an instant in 70 per cent. alcohol.

3. Stain in the alcohol-acetone solution of scarlet red for two to five minutes.

4. Wash quickly in 70 per cent. alcohol and transfer to water.

5. Counterstain in alum hematoxylin.

6. Wash thoroughly in water

7. Mount in glycerin or glycerin jelly.

The staining should always be done in a tightly stoppered bottle, because with any evaporation of the alcohol and acetone a precipitation of the staining material immediately takes place.

If, after staining with alum-hematoxylin, the sections are put into a 1 per cent. aqueous solution of acetic acid for three to five minutes and then washed thoroughly in tap water, the color of the nuclei is a clearer blue, in better contrast with the red color of the fat, and the staining is sharper.

With scarlet red and with Sudan III neutral fats stain darkest; then follow cholesterine esters and cholesterine-fatty acid mixtures. Other lipins stain lightly or not at all.

The Nile Blue Sulphate Stain for Fat (Lorrain Smith).— This dye is not so good or intense a general stain for fat as scarlet red, but it has certain valuable properties. For example, it gives a double stain, coloring neutral fats red, cholesterine ester and cholesterine-fatty acid mixtures reddish, sphingomyelin, cerebrosides and kephalin light bluish, fatty acids and soaps deep blue. Often there are shades between red and blue owing to the presence of lipoids. The danger of loss of fat is negative because the dye is used in an aqueous solution. It has been recommended for staining lipochrome (fat colored with the pigment carotin) in order to distinguish it from melanin, often present in the same cells. The lipochrome is colored intensely blue while the melanin, owing to its brown granules taking up a slight amount of blue, appears green. In a scarlet red stain the melanin is colored deeply enough red to render distinction from lipochrome difficult.

Directions.—1. Stain frozen sections of fresh or of formaldehyde-fixed tissue in a saturated aqueous solution of Nile blue sulphate for five to ten minutes.

2. Wash thoroughly in water.

3. Mount in glycerin or in glycerin jelly.

Schmorl recommends differentiation of the stained sections in a 1 per cent. aqueous solution of acetic acid for a few seconds followed by thorough washing in water in order to sharpen the stain and prevent diffusion of dye in the mounting medium.

Neutral red stains phosphatides, cerebrosides, fatty acids and soaps and is occasionally of value in demonstrating them.

Weigert's stain for the medullary nerve sheath is a method devised empirically for staining the fats contained therein. The principle involved has been extended so that a larger group of lipoids can be demonstrated by a modification of the method.

The stains for acid-fast bacilli probably depend on the presence of waxes contained in the bodies of the organisms.

Fischler's Method for Staining Fatty Acid Crystals and Soaps is a modification of Benda's stain for fat necroses and is based on the observation that fatty acid crystals and their calcium salts, after mordanting with acetate of copper, will form with hematoxylin a black lake which is almost insoluble in Weigert's differentiating mixture.

Directions.—1. Fix in formaldehyde.

2. Cut frozen sections.

3. Mordant the sections in a concentrated solution of copper acetate in water for two to twenty-four hours in the cold.

4. Wash in distilled water.

5. Stain for at least twenty minutes in a mixture of the two following solutions:

(*a*) Hematoxylin,	1 gm.;
Absolute alcohol,	10 c.c.;
(*b*) Concentrated aqueous solution of	
Carbonate of lithium,	1 c.c.
Distilled water,	9 "

The combined solution must ripen for several days before use.

6. Differentiate in Weigert's borax-ferricyanide of potassium mixture, greatly diluted, until the red blood corpuscles are decolorized.

7. Wash thoroughly in water.

8. Alcohol; xylol; colophonium.

The fatty acids are stained deep blue black as are also iron and calcium, but the latter two substances can be readily distinguished by their own reactions. If it is desired to stain the neutral fat with scarlet red, proceed as follows after step 7:

8. Stain in the scarlet red solution several minutes.

9. Pass quickly through 70 per cent. alcohol.

10. Wash in water.

11. Mount in glycerin jelly.

Inasmuch as the sodium and potassium salts of the fatty acids (namely, soaps) are soluble in formalin they must be transformed into the insoluble calcium soap by saturating the fixing solution with calcium salicylicum. By comparing stained sections of material fixed both ways it is possible to judge how much, if any, soap was present in addition to the fatty acids.

Calcium soaps are not soluble in a mixture of alcohol and ether, equal parts, or in simple hydrochloric acid, but in a combination of the two. Consequently they can be differentiated on the one hand from fatty acids which are soluble in the alcohol-ether mixture, or from calcium which is soluble in the simple acid. Besides fatty acids and soaps the method stains cholesterine oleic acid-glycerin-ester mixtures.

Ciaccio's Method of Staining Lipoids (positive for kephalin, kephalin mixtures, fatty acids, and soaps).

Directions.—1. Place very thin slices of fresh or of formaldehyde-fixed tissue in the following solution for two days:

5 per cent. aqueous solution of bichromate of potassium,	80 c.c.
Formalin,	20 "
Acetic acid,	10-15 drops.

2. Transfer to a 3 per cent. aqueous solution of bichromate of potassium for five to eight days.

3. Wash in running water twenty-four hours.

4. Dehydrate, clear in xylol and embed in paraffin, cut sections and return to alcohol.

5. Stain in a saturated solution of Sudan III or of scarlet red in 70 per cent. alcohol, in the incubator at 37° C. for one hour.

6. Wash off quickly in 50–70 per cent. alcohol.

7. Wash in water.

8. Counterstain in alum hematoxylin.

9. Wash in water.

10. Mount in the Apathy-Kasarinoff gum solution:

Gum arabic,	50 gms.
Cane sugar,	20 "
Water,	50 c.c.
Thymol,	0.05 gm.

Filter in oven at 55° C.

The lipoids which stain are colored orange red; the medullary sheaths of nerves bright red.

If Nile blue sulphate is used instead of Sudan III, the lipoids are stained blue-violet.

After step 2 it is possible to stain the neutral fats with osmic acid; they show up well by contrast with the red.

Lorrain Smith–Dietrich Stain for Lipoids.—The method is based on Weigert's stain for the medullary sheath which consists chiefly of glycolipins (cerebrosides), phospholipins and cholesterol. The method is positive for phosphatides (sphingomyelin, kephalin), cholesterine-fatty acid mixtures, cerebrosides, and to some extent for fatty acids and soaps.

Directions.—1. Fix in formaldehyde.

2. Cut frozen sections.

3. Mordant in a saturated aqueous solution of bichromate of potassium for twenty-four to forty-eight hours.

4. Wash quickly in water.

5. Stain for four or five hours in Kultschitzky's hematoxylin solution.

Hematoxylin,	1 gm.
Alcohol,	10 c.c.
Glacial acetic acid,	2 "
Water,	ad 100 "

6. Differentiate in Weigert's borax-ferricyanide of potassium solution.

7. Wash in water.

8. Examine in levulose syrup.

Cholesterol, which is of frequent occurrence in a variety of chronic lesions, forms characteristic large and small rhomboid crystals. They are soluble in acetic acid, alcohol, ether, xylol chloroform, etc.

The best known reaction for cholesterol is with iodine and sulphuric acid. Treated with iodine the crystals stain brown. On the addition of sulphuric acid they turn blue-violet, sometimes green, and finally red. The reaction may be followed under the microscope.

Treated with strong sulphuric acid only, the crystals, especially if slightly heated, turn yellow and then brownish red.

Golodetz recommends applying, instead of the strong acid, a mixture of five parts of sulphuric acid to two parts of 30 per cent. formalin.

Certain fats and fatty acids which react in the same way as cholesterol can be distinguished from it because they are stainable with osmic acid.

Cholesterines (cholesterine esters of the fatty acids) are doubly refractive when cold but not when warmed. After fixation, and when fresh preparations are chilled, they easily crystallize. On light warming they turn into double refractive droplets, but lose this appearance if heated more strongly.

Cholesterines are stained reddish by Nile blue sulphate and yellowish red by Sudan III. They are not very soluble in fat solvents.

Versé recommends the following method for obtaining the double refractive substances out of organs which contain but little of them. Tease apart small bits of tissue on a slide and then put over them a cover-slip. Run a little of an alcohol-ether mixture, equal parts, under an edge and after a short time a small drop of concentrated sulphuric acid. If much double refractive substance is present, observation with polarized light will show an active lighting up of fine droplets at the junction of the two fluids where before all was dark. If little of the substance is present it will be necessary to wait some time before the phenomenon appears.

Double refractiveness is exhibited by cholesterine esters and by cholesterine-fatty acid mixtures. It disappears on warming but returns on cooling. Sphingomyelin, cerebrosides and kephalin-cholesterine mixtures likewise show double refraction but it does not disappear on slight warming. Doubtful double refraction is exhibited by soaps, none by the neutral fats or fatty acids.

Lipochrome and Lipofuscin are names applied to fats colored by carotin dissolved in them; they occur in liver and nerve cells and in cardiac muscle fibers under a variety of pathologic conditions. The yellow pigment carotin is derived from ingested plant food, such as carots and squash.

Myelin, a term applied by the morphologists to the lipins forming the medullary sheath of nerves, is restricted by chemists to fat-like substances which arise within necrotic cells in the living body (necrobiotic) or post-mortem as the result of autolysis. They stain with neutral red—at least on warming—and positively by the Smith-Dietrich method.

Necrosis.—Necrosis in tissues is generally recognized by two features: either by the disappearance of the nuclei, although the cell-outlines may be visible, so that the nuclear stain is no longer possible, or by the presence of irregular, larger or smaller masses, generally supposed to be due to a fragmentation or breaking-up of the chromatin, which stain intensely with nuclear stains. The disappearance of the nucleus is not synchronous with the death of the cell, but

begins some twenty-four hours later, so that it is really evidence of changes following necrosis. It follows from the above that the microscopic evidence of necrosis is best studied in sections of tissues hardened in fixatives which favor nuclear staining, such as Zenker's fluid, formaldehyde, etc. Teased preparations and frozen sections of fresh tissue are much less useful.

For the study of sections from hardened material double stains with alum-hematoxylin and eosin, or, still better, with eosin followed by Unna's alkaline methylene-blue solution, after Zenker's fixation, are very useful, for the reason that the necrotic areas usually stain rather deeply with the diffuse stain, and are thereby brought out sharply. This is particularly true of necroses of the liver.

For rendering the fragmented nuclei prominent the same methods may be followed as for mitosis. A fuchsin stain washed out by picric acid in the alcohol will often give excellent results.

Caseation is probably a tissue-change following local necrosis. Macroscopically and microscopically it resembles harder or softer cheese. Under the microscope it appears as coarsely or finely granular masses which have more or less completely lost the original tissue-structure. The chemical changes which have taken place have not been studied. Fibrin may or may not be present. Caseous tissue possesses no peculiar staining reactions. Fragmented nuclei are frequently present in it, especially in the peripheries of the areas.

Fibrin.—Fibrin usually appears as delicate, transparent, slightly refractive threads which are often closely matted together so as to form large masses. More rarely it appears as coherent masses of the finest granules, as homogeneous glassy lumps, or as thin sheets. The characteristic reaction for fresh fibrin is that it quickly swells up and optically dissolves in very dilute acetic acid.

Fibrin is well brought out in sections of hardened tissues by a double stain of alum-hematoxylin and eosin, or of eosin followed by Unna's alkaline methylene-blue solution, especially

if the specimens have been fixed in Zenker's fluid. Two other stains which bring it out with great sharpness are phospho-tungstic-acid-hematoxylin and the aniline-blue method for collagen fibrils.

Weigert's Differential Stain for Fibrin.—1. Harden in alcohol, embed in paraffin and cut sections.

2. Stain in lithium carmine (see page 73).

3. Stain in Weigert's aniline-methyl-violet about 15 seconds.

4. Wash off with normal salt solution.

5. Weigert's iodine solution 15 to 20 seconds.

6. Wash off with water.

7. Blot and decolorize in aniline oil and xylol equal parts.

8. Wash off with three changes of xylol.

9. Xylol colophonium or balsam.

To stain sections fixed in formaldehyde or in a chrome salt, place them in a $\frac{1}{3}$ per cent. aqueous solution of permanganate of potassium for ten minutes, wash in water, and reduce in a 5 per cent. aqueous solution of oxalic acid for two to three hours or longer. Then wash thoroughly in water.

The fibrin and those bacteria which are stained by Gram are stained blue. The nuclei are red if the decolorization is carried far enough. It can easily be watched under the low power of the microscope. The method is not always successful, especially with tissues which are old. Besides the fibrin, certain forms of hyaline are often stained by this method.

Differential stains for fibrin are also obtained by the chloride of iron hematoxylin stain (page 96) and by the connective-tissue stain (page 118). The former is applicable after any fixing reagent, the latter only after Zenker's fluid.

The phosphotungstic-acid-hematoxylin method is also useful if all the steps are followed out as for neuroglia fibrils.

Mucin.—The term "mucin" is applied to a proteid sub-stance having certain chemical reactions, and also to certain other substances which give the same reactions, but do not belong to the proteids. These various substances of secretory and degenerative origin cannot be distinguished microscopically, and have been investigated but little chemically. The reac-

tions in common are the following: they dissolve in water to form a slimy fluid; they are precipitated from slightly alkaline solutions by acetic acid; the fresh precipitate dissolves in alkalies and in neutral salt solutions.   Acetic acid, usually employed for this purpose in a 1 or 2 per cent. solution, precipitates mucin in the form of threads or granules.   This reaction with fresh tissues has long been the main test for mucin.   The acetic acid is drawn under the cover-slip by means of filter-paper placed at the opposite edge.   The preparation should be mounted in water, not in salt solution which may hinder or entirely prevent the reaction from taking place.   Of late certain color reactions have become prominent.   Mucin is coagulated into threads by alcohol or corrosive sublimate, and in this form can be stained by a number of staining reagents. Alum-hematoxylin under certain conditions will stain mucin. According to P. Mayer, these conditions depend on a certain degree of ripeness of the solution, on the presence of enough alum to keep the nuclei from staining deeply, and, most important of all, on the absence of any free acid.   This is difficult to manage, unless the solution is carefully neutralized, on account of the acid properties of alum.   Mayer, therefore, recommends staining the sections in muchematein.

Mayer's Muchematein.—

Hematein,	0.2	gm;
Chlorid of aluminum,	0.1	"
Glycerin,	40	c.c.;
Water,	60	"

Rub up the hematein with a few drops of glycerin and the chlorid of aluminum, and dissolve the mixture in the glycerin and water.   Mucin appears blue; the other tissue elements are not stained.

Various aniline dyes have been recommended for staining mucin: those most favorably spoken of are methylene-blue (Orth), Bismarck brown (P. Mayer), thionin (Hoyer), polychrome methylene-blue (Unna), and toluidin-blue.   The drawback to most of the aniline stains is that they are quickly

extracted by the alcohol used for dehydrating. On this account P. Mayer highly recommends Bismarck brown, because permanent mounts can be easily made with it. It is not extracted by alcohol, and it does not fade in Canada balsam like many of the others.

Hardening in corrosive sublimate and embedding in paraffin are generally recommended as preferable to hardening in alcohol and embedding in celloidin. Stain sections for five to fifteen minutes in a rather dilute aqueous solution of the dye chosen. Of Bismarck brown use a saturated aqueous solution, and stain, if necessary, twenty-four hours. With thionin, toluidin-blue, and polychrome methylene-blue metachromatic stains are obtained; the mucin is colored red, the rest of the tissue blue. Two special methods for staining mucin are given in detail:

Hoyer's Thionin Stain.—Mucin, red; everything else, blue.

1. Harden in corrosive sublimate, followed by alcohol.

2. Paraffin sections are passed through xylol, chloroform, and 95 per cent. alcohol to free them from paraffin, and are then placed in a 5 per cent. aqueous solution of corrosive sublimate for three to five minutes.

3. Stain in a dilute solution of thionin for ten to fifteen minutes.

4. Alcohol.

5. Clear in the mixture of the oils of cloves and thyme.

6. Turpentine oil or oil of cedar.

7. Xylol colophonium or balsam.

Before the staining the sections must not be treated with iodine solution to get rid of the precipitate of mercury, because it spoils the staining.

Unna's Polychrome Methylene-blue Stain.—1. Fix in alcohol. Stain paraffin or celloidin sections in polychrome methylene-blue five to ten minutes or longer.

2. Wash in acidulated water.

3. Fix in 10 per cent. solution of bichomate of potassium half a minute.

4. Wash in water.

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5. Dry on slide with filter-paper.

6. Decolorize in aniline plus 1 per cent. hydrochloric acid (a few seconds only).

7. Wash off with oil of bergamot.

8. Xylol colophonium or balsam.

Nuclei blue, mucin, cartilage, and amyloid red.

Pseudo=mucin dissolves in water to form a slimy material, and is precipitated from its solutions by alcohol in thread-like masses which are again soluble in water. It is not affected by acetic acid. Pseudo-mucin is found in certain ovarian and other tumors.

Colloid and Hyaline.—The terms colloid and hyaline are not yet sharply limited to definite chemical substances. The term colloid was originally applied to the homogeneous substance found in the thyroid gland, but has been broadened to include various substances of a similar appearance. The term hyaline is still more indefinite, but its use may be said to be applied most generally to those homogeneous substances which stain deeply with various stains, in contradistinction to those which, like colloid, show no marked affinity for staining reagents after ordinary fixatives.

Unquestionably, numerous substances of different chemical composition and of varying origin have been grouped under these two titles because of their physical and optical characteristics—namely, that they occur as glassy, refractive, homogeneous, occasionally colored gelatinous or firm masses. Chemically, very little that is definite is known about them, and they possess no peculiar chemical reactions. Several attempts have been made to classify them in accordance with their reactions to various staining reagents.

Von Recklinghausen applied the term colloid to all the homogeneous, transparent-looking substances, including mucin, amyloid, etc., and reserved the term hyaline for a special group, which, according to him, is characterized by the following peculiarities: it resembles amyloid in physical characteristics, but does not react to iodine; it stains deeply with acid dyes, such as eosin and acid fuchsin.

Ernst has recently endeavored to differentiate two groups of hyaline substances, colloid and hyaline, by means of their reaction to Van Gieson's picro-acid fuchsin solution. According to him, true hyaline stains with acid fuchsin alone, and appears of a deep-red color, while colloid, of which the typical example is found in the thyroid gland, stains with both picric acid and acid fuchsin, so that it appears of an orange or yellowish-brown color. He has also tried to prove that all colloid is derived from epithelial cells, while all hyaline comes from connective tissue or from blood-vessels.

According to Von Kahlden, these differential staining reactions with Van Gieson's mixture claimed by Ernst for colloid and hyaline are by no means justifiable, because true colloid often stains a deep red. Furthermore, Unna has shown that in the skin connective-tissue cells can give rise to the so-called true hyaline, of which part is acidophilic and part basophilic, while the intercellular substance gives rise to colloid.

The last attempt to classify the various homogeneous substances on the basis of their reactions to dyes, apparently the only method possible at present, has been made by Pianese as a result of his studies of the various degenerative processes occurring in cancer-cells. He used a special fixative (see p. 49) and five different staining methods (see p. 82, methods III. A. and B., IV., V., and VI.). Of these methods, III. B. is the best, because it gives a characteristic color to each substance—hyaline, brick-red; colloid, bright green; mucin, clear sky-blue; and a substance resembling amyloid, a dark reddish-violet. Besides these distinct reactions for colloid, hyaline, mucin, and a substance resembling amyloid, he found others less definite; one of these he calls pseudo-mucin and another pseudo-colloid. As a basis for his studies he took the reactions of amyloid, mucin (intestine), colloid (thyroid gland), and hyaline (hyaline remains of ovarian follicles, hyaline degeneration of renal glomeruli), with the same stains after fixation in his own hardening mixture.

The above brief historical statement is considered necessary to show the present views in regard to these various,

more or less indefinite, homogeneous, transparent substances. For demonstrating them after the usual hardening reagents, of which alcohol and corrosive sublimate are perhaps the best, a double stain with alum-hematoxylin and eosin is very useful. Certain of the homogeneous substances stain deeply with eosin; others, like the transparent drops and masses occasionally found in the walls of the blood-vessels of the brain, stain with hematoxylin. Sometimes good results can be obtained with Weigert's fibrin stain or with carbol-fuchsin. The most generally useful stain, aside from alum-hematoxylin and eosin, is probably Van Gieson's mixture.

The hyaline in liver-cells in alcoholic cirrhosis stains deeply by the eosin–methylene-blue method after fixation in Zenker's fluid. The color varies from blue to red, depending, to some extent at least, on the freshness of the tissue. It stains deep blue in phosphotungstic-acid-hematoxylin preparations.

Van Giesons Stain.—1. Stain deeply in alum-hematoxylin.

2. Wash in water.

3. Stain three to five minutes in a saturated aqueous solution of picric acid, to which is added enough of a saturated aqueous solution of acid fuchsin to give it a deep-red color. The effect of various proportions is sometimes useful.

4. Wash in water.

5. Alcohol.

6. Oleum origani cretici.

7. Xylol colophonium or balsam.

The transparent homogeneous substances usually stain from orange to deep red in color; connective tissue, red.

Unna's Method for Hyaline and Colloid Material.—*A.* Harden in alcohol. 1. Acid fuchsin (2 per cent. aqueous solution) five minutes.

2. Saturated aqueous solution of picric acid two minutes.

3. Saturated alcoholic solution of picric acid two minutes.

4. Wash off in alcohol.

5. Oil, xylol colophonium or balsam.

Hyaline and connective-tissue fibers, red; colloid of thyroid gland, yellow; cytoplasm, yellow.

B. To show acidophilic and basophilic hyaline: 1. Water-blue (2 per cent. aqueous solution), twenty to thirty seconds.

2. Water.

3. Carbol-fuchsin one to two minutes.

4. Water.

5. Alcohol slightly tinged with iodine.

6. Pure alcohol.

7. Oil, xylol colophonium or balsam.

Nuclei, keratin, and large hyaline masses, cherry red; connective-tissue fibrillæ, cytoplasm, and small hyaline bodies, blue.

For finer work the methods of Pianese should be used.

Keratohyalin (*Unna*).—1. Stain sections in a fairly old alum-hematoxylin solution until they are over-stained.

2. Place in a very weak solution of permanganate of potassium (about 1:2000) for ten seconds.

3. Dehydrate and decolorize in alcohol.

4. Oil, balsam.

An isolated stain of the granules of keratohyalin is obtained, blue-black in color.

In like manner a 33 per cent. solution of sulphate of iron acting for ten minutes, or a 10 per cent. solution of chlorid of iron for a few seconds, will produce the same effect. Ordinarily, sections are stained deeply in alum-hematoxylin, and decolorized with acetic acid and alcohol or with hydrochloric acid and alcohol.

Glycogen.—Glycogen is a carbohydrate of slightly varying composition, occurring in cells and nuclei, more rarely in the intercellular tissue, either diffusely or more commonly in the form of larger and smaller masses and granules of a transparent homogeneous appearance. It is demonstrated microchemically by means of its reaction with iodine, which stains it brown. It is easily differentiated from amyloid by the fact that with the exception of the glycogen from certain sources, such as cartilage-cells, it is readily soluble in water and does not give the iodine-sulphuric-acid reaction.

In consequence of its property of dissolving readily in water the aqueous Lugol's solution of iodine cannot be employed for

staining glycogen in fresh tissues. Instead, a thick solution of gum arabic containing 1 per cent. of Lugol's solution must be used, or, better still, equal parts of glycerin and Lugol's solution, in which the sections are more perfectly cleared.

For sections hardened in absolute alcohol the same methods may be used, but better results, and practically permanent mounts, can be obtained by the method of Langhans. Lugol's solution is used for staining the sections, because after hardening in alcohol the glycogen is much less soluble in water than in the fresh state. The iodine-glycerin mixture would probably be better. Best's carmine stain, however, affords, by all odds, the most brilliant, permanent and satisfactory method of demonstrating glycogen in the tissues, and has practically superseded the use of iodine in any way.

1. Langhans' Iodine Stain.

1. Stain paraffin sections in Lugol's solution.

2. Dehydrate in 1 part of tincture of iodine to 3 or 4 parts of absolute alcohol.

3. Clear in oleum origani cretici.

The sections are to be preserved in oil. Even a ring of balsam around the cover-slip will cause the color to fade. Other oils are not so good.

2. Lubarsch's Iodine-Hematoxylin Stain.

1. Fixation in absolute alcohol.

2. Stain paraffin sections for five minutes in the following solution, which should be filtered and carefully protected from sunlight:

Delafield's hematoxylin,	2 parts
Lugol's solution,	2 "
Water,	1 part

3. Absolute alcohol, xylol, xylol balsam.

4. Expose to daylight one to two days. Glycogen brown, nuclei blue.

Lubarsch's Gentian-violet Stain.—Alcohol fixation; paraffin embedding.

1. Stain with Meyer's alcoholic carmine solution, differentiate in acid alcohol, wash off in absolute alcohol.

2. Stain in aniline oil gentian-violet for one to two minutes, warming slightly if necessary

3. Wash quickly in water.

4. Gram's solution of iodine on section continuously for five to ten seconds.

5. Dry thoroughly with filter-paper.

6. Dehydrate and differentiate in aniline-oil xylol (2 to 1) or in pure aniline oil.

7. Wash thoroughly in xylol and mount in balsam.

Nuclei red; glycogen dark blue to violet. It is advisable to expose the sections to daylight for one to two days. The preparations will keep up to one year.

Best's Carmine Stain.—Fix tissues in alcohol: formaldehyde and corrosive sublimate are not so good.

Embed in celloidin, which prevents the glycogen from dissolving in water. Paraffin and frozen sections should not be used.

The stock carmine solution is made as follows:

Carmine,	2.0 gms.;
Potassium carbonate,	1.0 gm.;
Potassium chloride (KCl),	5.0 gms.;
Aq. dest.,	60.0 c.c.

Boil gently and cautiously for several minutes.
After cooling add—

Liq. ammon. caustic.,	20.0 c.c.

In tightly stoppered bottles this solution will keep and be available for staining glycogen for two months in winter, and for about three weeks in summer.

The staining method is as follows:

1. Stain sections deeply with alum-hematoxylin.

2. Decolorize with acid alcohol, if necessary.

3. Wash thoroughly in running water.

4. Stain sections for five minutes or longer in the following solution:

The above carmine solution (freshly filtered),	2.0 c.c.;
Liq. ammon. caustic.,	3.0 "
Methyl alcohol,	3.0 "

5. Differentiate in—

Alcohol abs.,	80.0 c.c.;
Methyl alcohol,	40.0 "
Aq. dest.,	100.0 "

from three to five minutes, changing the fluid occasionally until it remains uncolored.

6. Wash off in 80 per cent. alcohol.

7. Alcohol, oil, xylol colophonium or balsam.

Glycogen red, nuclei blue. The method also stains the peptic cells of the stomach, the corpora amylacea of the nervous system, and sometimes the mucin in goblet-cells and the granules of mast-cells.

Caution: Do not put the sections into water after steps 4 or 5, because the carmine will diffuse out of the specimens.

Amyloid Infiltration.—Amyloid is a combination of an albuminous body with chondroitin sulphuric acid. It is insoluble in water, alcohol, ether, and dilute acids, and is not digested by pepsin and hydrochloric acid. It is distinguished from the other homogeneous substances, except glycogen, by the fact that it is stained mahogany-brown by iodine in solution. The reaction is particularly useful as a test on fresh gross material. If a section containing amyloid be quickly and lightly stained in Gram's iodine solution and then transferred to sulphuric acid, the color of the amyloid will usually change at once or in a few minutes from red, through violet, to blue. Sometimes the color turns simply of a deeper brown. Several of the aniline dyes give almost as perfect characteristic color reactions for amyloid as iodine, and are perhaps better for the purposes of histological study. Any of these differential stains may be used with fresh or hardened

tissues. Alcohol as a hardening reagent gives the best results, but the other fixatives may be employed. Unfortunately, good permanent mounts cannot be made with any of the characteristic stains, so that the ordinary double stains of alum-hematoxylin with eosin or Van Gieson's mixture will often be found of the greatest help in studying the distribution of amyloid. The aniline-blue connective-tissue stain can also be highly recommended because it stains amyloid light blue, so that it stands out in marked contrast to the red of the liver-cells.

Iodine Reaction for Amyloid.—1. Stain sections in a weak solution of iodine (Lugol's solution diluted until of a clear yellow color) for three minutes.

2. Wash in water.

3. Mount and examine in water or glycerin.

If the tissue reacts strongly alkaline, a condition which may result from post-mortem decomposition, the color reaction with iodine will not take place. In such cases the tissue or the sections of it should be treated with dilute acetic acid before applying the test. The normal reaction of amyloid with iodine may be increased by treating the section after staining with dilute acetic acid.

Langhans' Method for Obtaining Permanent Mounts with Iodine.—1. Harden in alcohol and stain in Mayer's alcoholic carmine solution.

2. Stain sections in Lugol's solution five to ten minutes.

3. Dehydrate quickly in 1 part of tincture of iodine to 3 or 4 parts of absolute alcohol.

4. Clear and mount in oleum origani cretici.

The color is said to keep remarkably well. Other oils or balsam cause it to fade quickly. The staining in Lugol's solution may be omitted, as the tincture of iodine usually stains the amyloid sufficiently deeply.

Iodine and Sulphuric-acid Reaction.—1. Stain quickly and lightly in dilute Lugol's solution.

2. Treat with sulphuric acid, either concentrated or dilute (1 to 5 per cent.), on the slide or in the staining dish. Strong hydrochloric acid may be used in the same way.

The change of colors from red to blue already spoken of usually occurs within a few minutes, but occasionally does not take place at all.

The following substances give reactions with the above iodine tests:

1. *Cholesterin crystals* are stained rather dark with dilute iodine solution, and turn a beautiful blue color at the edges on the addition of strong sulphuric acid.

2. The *corpora amylacea* in the prostate and central nervous system stain brown with the dilute iodine solution.

3. *Starch-granules* stain blue with dilute iodine solution.

4. *Cellulose* stains yellow with iodine. If washed and treated with strong sulphuric acid, it turns blue where the acid touches it.

For the reactions with the aniline dyes the sections must be free from celloidin.

Reaction with Methyl-violet.—1. Stain frozen sections of fresh or of formaldehyde or alcohol fixed tissue in 1 per cent. methyl-violet three to five minutes.

2. Wash in a 1 per cent. aqueous solution of acetic acd.

3. Wash thoroughly in water to remove all trace of acid.

4. Examine in water or in glycerin.

The stain will keep for some time if mounted in a saturated solution of acetate of potash or in levulose. Other methods are to stain in aniline-methyl-violet and to wash out in a 1 per cent. solution of hydrochloric acid, or to stain in a strong solution of methyl-violet to which acetic acid is added, and to wash out in water. The amyloid is stained violet-red, the tissue blue. Sections of tissues embedded in celloidin will not give the reaction unless the celloidin is removed. The color reaction shows best when the light for the microscope is taken from a white cloud, not from the blue sky.

Reaction with Iodine-green.—1. Stain fresh or hardened sections in a $\frac{1}{3}$ per cent. aqueous solution of iodine-green for twenty-four hours.

2. Wash in water.

3. Mount in water or glycerin.

Amyloid, violet-red; tissue, green. Stilling claims that the reaction is surer than with methyl-violet.

Reaction with Bismarck Brown and Methyl-violet (Birch-Hirschfeld).—1. Stain in a 2 per cent. alcoholic solution of Bismarck brown for five minutes.

2. Wash in absolute alcohol.

3. Wash in distilled water ten minutes.

4. Stain in a 2 per cent. aqueous solution of methyl-violet five to ten minutes.

5. Wash in dilute acetic-acid solution.

6. Wash thoroughly in tap water.

7. Mount in levulose.

Amyloid, red; tissue, brown.

Mayer's Stain for Amyloid.

1. Transfer paraffin sections without previous treatment directly from the knife to a warmed (40° C.) ½ per cent. aqueous solution of gentian-violet for five to ten minutes.

2. Wash in water and differentiate in a 1 per cent. solution of acetic acid for ten to fifteen minutes.

3. Wash thoroughly in water.

4. Transfer to a half concentrated aqueous solution of alum. Wash off in water.

5. Transfer sections to slide and allow the water to evaporate.

6. Remove paraffin and clear with xylol. Mount in xylol colophonium or balsam.

Pigments.—The various pigments found in the human body under normal and pathological conditions are commonly divided into three groups:

1. *Hematogenous pigments*, derived from the coloring matter of the blood.

2. *Autochthonous pigments*, formed by cells from colorless elements of nutrition.

3. *Exogenous pigments*, introduced into the body from without.

1. **Hematogenous pigments** occur in considerable variety. Some of them, like hemoglobin, contain iron but the latter is too intimately bound up to the protein group in the molecule

to react to the ordinary tests. From the pathological point of view hemosiderin is the most important of these pigments which are listed below with their chief characteristics and staining reactions.

(a) *Hemoglobin and Methemoglobin.*—Soluble in water and in alcohol not absolute; occur as yellowish to yellowish brown granules and droplets (which in hemoglobinuria may be compacted into casts); in the fresh condition but not after fixation stain bright red on treatment with potassium hydrate, sodium carbonate or prussic acid. For fixation use the same solutions as for the preservation of red blood corpuscles, namely, Zenker's fluid, formaldehyde, corrosive sublimate. The granules will stain intensely with eosin.

(b) *Parhemoglobin.*—A form of hemoglobin, crystallizes like it but is insoluble in alcohol.

(c) *Sulphemoglobin.*—A greenish compound of sulphur and methemoglobin to which the greenish color frequently seen in the abdominal wall of cadavers is due; may occur in the blood during life (sulphemoglobinemia); diagnosis made by spectroscopic examination of the blood; must be distinguished from methemoglobin.

(d) *Hematin.*—Occurs as an amorphous, dark brown or bluish black material in old extravasations of blood; not often found. It has recently been stated by Brown that the pigment produced by the malarial parasites is hematin.

(e) *Hemofuscin.*—Light yellow granules which stain intensely with certain aniline dyes, especially basic fuchsin and methylene blue; do not give the iron reaction; are insoluble in peroxide of hydrogen; are found in the liver and certain other tissue in hemochromatosis; are well preserved in all ordinary fixatives.

Fuchsin Stain for Hemofuscin (Mallory):

Directions:

Fixation: Zenker's fluid, alcohol or formaldehyde.

1. Stain paraffin sections in alum-hematoxylin until the nuclei stand out sharply defined.

2. Wash in water.

3. Stain for five to twenty minutes in the following solution:

Fuchsin,	0.1–0.5 gram;
Alcohol, 95 per cent.,	50.0 c.c.;
Water,	50.0 "

4. Wash off in water.

5. Differentiate and dehydrate in 95 per cent. followed by absolute alcohol.

6. Xylol, xylol colophonium.

Nuclei blue, hemofuscin granules bright red, hemosiderin unstained.

The method can be used equally well on celloidin sections if oleum origani cretici is employed in place of xylol.

The stain sharply differentiates the granules of hemofuscin from those of hemosiderin but no other claim is made for it except that it does not stain melanin.

(*f*) *Hemozoin* (Sambon), (hematin according to Brown).— A black pigment occurring as granules; formed only by the action of the malarial parasites living on or in the red blood corpuscles; can be distinguished from carbon by its solubility in concentrated sulphuric acid; does not give iron reactions.

(*g*) *Hemosiderin.*—Occurs as bright colored, yellowish brown and brown granules and masses; is insoluble in water, alcohol, ether, and alkalies, but is soluble in acids; does not stain with fat stains; gives reactions for iron both in the fresh state and after fixation. The best fixative for tissues containing hemosiderin is stated to be alcohol; the alcohol-formaldehyde mixture comes next, formaldehyde third. Corrosive sublimate solution without the addition of acetic acid may be used. The use of fixatives containing chrome salts is advised against and yet perfect results can be obtained after fixation in Zenker's fluid as will be stated below. The fault lies not in the fixation but in the method of performing the test. Hemosiderin tends to diffuse in the tissues as the result of post-mortem changes, hence it is important to obtain tissues as fresh as possible and to cut them into thin sections for fixation.

The reaction with ammonium sulphide to form the greenish black sulphide of iron has the disadvantage of not being diagnostic; certain other metals such as silver, lead and mercury give similar black reactions; but when other metals can be excluded, as in hemochromatosis, the reaction is of great value because it takes place without solution of the pigment granules which remain unaltered in form though changed in color. The method is of great value when photomicrographs of the stained sections are desired. An additional advantage is that the ammonium sulphide reacts with both ferric and ferrous compounds. The original methods of performing the iron reactions have been frequently modified, since they were first introduced by Quincke and Perle, because no one of them is perfectly satisfactory. The methods given here are believed to be, in some respects at least, distinct improvements.

A. **Iron Reaction with Ammonium Sulphide** (**Mallory**). *Directions*.—Fixation in Zenker's fluid, alcohol or formaldehyde.

1. Stain paraffin sections in alum-hematoxylin until the nuclei are of a deep blue color.

2. Wash in water.

3. Place in a mixture of one part of strong yellow ammonium sulphide to three parts of 95 per cent. alcohol for one to two hours or longer. Use a glass staining dish with tightly fitting cover to prevent evaporation of the ammonia. The alcohol serves two purposes: it prevents paraffin sections from dissolving off the slide, and protects celloidin sections from wrinkling.

4. Wash quickly but thoroughly in several changes of water.

5. Dehydrate in 95 per cent. and absolute alcohol.

6. Xylol, xylol colophonium.

Nuclei blue, hemosiderin granules of an intense black. The stain keeps well. The results after Zenker fixation are perfect. If the sections are kept in water or alcohol too long the black color fades as the sulphide changes to the hydroxide.

The method is applicable also to celloidin and to frozen sections by using origanum oil as the clearing agent.

B. **Berlin Blue.**—Due to the action of ferrocyanide of potassium and hydrochloric acid on a ferric salt.

This is the classical diagnostic color reaction for iron due to the formation of Berlin blue. It has at least two drawbacks. Hemosiderin is soluble in the hydrochloric acid and the Berlin blue in the ferrocyanide of potassium As a result the stain readily diffuses, the fine granules all disappear and the larger ones have blue halos around them. A fine precipitate may be formed and deposited on the surfaces of the sections.

There are various methods of performing the test. The following is recommended for ordinary purposes.

Fixation in alcohol or formaldehyde. After Zenker's fluid the hemosiderin dissolves much too easily in the hydrochloric acid so that only a diffuse stain results.

Iron Reaction with Ferrocyanide of Potassium (Mallory). *Directions.*—1. Stain paraffin (or celloidin) sections for ten to twenty-five minutes in the following mixture:

Ferrocyanide of potassium, 2 per cent. aqueous solution, freshly prepared (not over one week old), 1 part;
Hydrochloric acid, 1 per cent. aqueous solution, 3 parts.

Much better results will be obtained if, following a suggestion by Maude E. Abbott, the reaction is hastened by high temperature. Heat the mixture in a test tube until beads of gas form on the inner surface of the glass (about 60–80° C.) and place the sections in it or pour it over them. The reaction takes place within thirty to forty-five seconds.

2. Wash immediately and thoroughly in several changes of water.

3. Counterstain in $\frac{1}{10}$ to $\frac{1}{2}$ per cent. solution of basic fuchsin in 50 per cent. alcohol for five to twenty minutes.

4. Wash off in water.

5. Differentiate and dehydrate in 95 per cent. followed by absolute alcohol.

6. Xylol, xylol colophonium.

Clear celloidin sections in origanum oil.

Nuclei and hemofuscin bright red, hemosiderin blue.

In the method recommended here the reaction takes place so completely owing to the excess of acid and so quickly owing

to the heat that there is little time for diffusion. The stain is intense and sharp even for the finest granules.

To Stain Gross Specimens Containing Iron (Mallory).— The ferrocyanide of potassium and hydrochloric acid reaction can easily be applied to slices of tissues in the fresh condition for diagnostic purposes or after fixation to make attractive and instructive preparations to use in teaching or to preserve in a medical museum.

1. Fix large slices of liver and other organs containing iron in Kaiserling solution No. 1 for several days.

2. Wash thoroughly in running water for twenty-four hours.

3. Preserve in 80 per cent. alcohol.

4. Cut into thin slices so as to obtain perfectly smooth surfaces.

5. Place the sections which it is desired to stain in a freshly prepared ferrocyanide of potassium and hydrochloric acid mixture for fifteen to thirty minutes, moving them about frequently.

6. Wash in running water for twenty-four hours or longer to remove all trace of chemicals.

7. Preserve and mount in 80 per cent. alcohol (not in Kaiserling solution No. 3 which causes the blue color to diffuse) along with unstained sections for contrast.

C. **Turnbull's Blue.**—The second diagnostic stain for iron is due to the action of ferricyanide of potassium and hydrochloric acid on a ferrous salt.

When hemosiderin is treated with ammonium sulphide the finest granules and the surface of the coarse ones are changed to ferrous sulphide. In consequence of this transformation, sections first stained with ammonium sulphide may be treated with ferrocyanide of potassium and hydrochloric acid (MacCallum, Hall, Nishimura) to form Berlin blue, or with ferricyanide of potassium and hydrochloric acid (Tirmann) to form Turnbull's blue, but neither method can stain all the iron present. Moreover, for the reasons already stated, both these methods are faulty and imperfect on account of the diffusion of the blue color owing to the action of the hydrochloric acid.

If, however, the acid is omitted in the second reaction the ferricyanide of potassium alone will give a perfect, deep blue stain after ammonium sulphide, without diffusion, provided its time of action is limited, which rivals the ammonium sulphide stain in sharpness and surpasses it in color and diagnostic value. The intensity of the blue stain depends on the length of action of the ammonium sulphide. If it is short, only the surface of the coarse granules is transformed to ferrous sulphide. When the color is turned blue the yellow within shows through the blue surface stain making the larger masses appear dark greenish. Therefore, it is important to have the reaction with ammonium sulphide as complete as possible. All the hemosiderin, so far as practicable, should be turned to a ferrous salt. The reaction is quicker and more effective and takes place even after Zenker fixation if 5 per cent. of acetic acid, which seems to have no dissolving effect on either hemosiderin or Turnbull's blue, is added to the ferricyanide of potassium.

Iron Reaction with Ammonium Sulphide and Ferricyanide of Potassium (Mallory). *Directions.*—Alcohol, formaldehyde or Zenker fixation.

1. Stain paraffin sections in

Strong yellow ammonium sulphide,	1 part;
Alcohol, 95 per cent.,	3 parts.

for one to two hours or longer (twelve to twenty-four hours for Zenker-fixed tissues).

2. Wash off in water.
3. Place in a freshly prepared mixture of

Ferricyanide of potassium (2 per cent. aqueous solution),	95 c.c.;
Glacial acetic acid,	5 "

for ten to twenty minutes or longer (twelve to twenty-four hours for Zenker-fixed tissues).

4. Wash thoroughly in several changes of water.
5. Counterstain sections in a $\frac{1}{10}$ to $\frac{1}{2}$ per cent. solution of basic fuchsin in 50 per cent. alcohol for five to twenty minutes.

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6. Wash off in water.

7. Differentiate and dehydrate in 95 per cent. followed by absolute alcohol.

8. Xylol, xylol colophonium.

Clear celloidin sections in origanum oil.

Nuclei and hemofuscin granules bright red, hemosiderin blue.

(*h*) *Hematoidin = bilirubin* its isomer: Occurs as orange colored or red rhombic plates or as radiating bunches of yellow needles; contains no iron; is insoluble in water, alcohol, ether; dissolves in chloroform; is found in old hemorrhages and especially in infarcts of the spleen and brain.

(*i*) *Bile pigment = bilirubin = hematoidin:* Insoluble in water, alcohol, ether; occurs as yellowish granules and masses; by oxidation is converted into the green biliverdin (this reaction takes place also when tissues containing bile are fixed in solutions containing potassium bichromate).

(*j*) *Hematoporphyrin =* hematin minus iron: Traces of it appear normally in the urine but the amount may be much increased in certain diseases and color the urine Burgundy red; is decomposed by concentrated sulphuric or nitric acid, with the same color reactions as bile pigment, but is not soluble in dilute acids or alkalies; does not bleach.

2. **Autochthonous** (**Autogenous**) **Pigments.**—The pigments produced in the body, aside from those of hematogenous origin, are grouped under the term *melanin.* They are closely related but not yet proved identical. They occur normally in hairs and in the deeper layers of the epidermis, most abundantly in people of dark complexion, in the chromatophores of the corium and pia, and in the eye. With advancing age they appear in various cells of the body (hepatic and nerve cells, cardiac muscle fibers).

Pathologically melanin is present abundantly in the chromatophores forming freckles and pigmented nevi and giving rise to the tumors known as melanoblastomas (chromatophoromas). When the tumor growth is very extensive the pigment may be excreted in the urine, usually in the form of melanogen. In Addison's disease, which results from destruction of the adrenal

glands, usually by tuberculosis, the deposit of melanin is greatly increased in the epidermis, liver, heart and brain.

Melanin is a dark brown amorphous substance of great coloring power which occurs in cells as brown to black granules. It contains no iron or fat and does not react to the iron tests, but is tinged slightly by the fat dyes (scarlet red and Nile blue sulphate). It is insoluble in ordinary reagents except alkalies and is very slowly bleached by sunlight and by oxidizing agents (peroxide of hydrogen, ferric chloride). It is stained black by the nitrate of silver methods (Bielschowsky, Levaditi).

3. **Exogenous Pigments.**—Various substances gain entrance to the body and are deposited there as pigments. The most important are carotin, carbon, iron and silver.

Carotin.—The principal one of the carotinoids is an orange yellow pigment derived from the carrot, the squash and certain other vegetables. It is easily decolorized by the action of strong sunlight and by oxidizing agents (peroxide of hydrogen, ferric chloride) and does not stain by the nitrate of silver methods. It is readily soluble in fats and in fat solvents, and is therefore on account of this latter property easily extracted from tissues by the action of alcohol, ether, chloroform. The yellow color of fats in the human body is due to it. If it is ingested in too large quantities it may color yellow the skin of infants or the conjunctivæ of adults suggesting jaundice.

Carotin exists in the carrot in granular form. It does not stain with the fat dye Nile blue sulphate. In the human body carotin is always dissolved in fat which does stain with this dye. Hence Nile blue sulphate is often used to differentiate this colored fat from melanin. The important point to realize is that the dye stains the fat but not the pigment.

Fat stained with carotin (lipochrome) often occurs in the same cells (liver, heart) with melanin and occasionally with hemosiderin; hence confusion arises if differential stains are not properly carried out and the results correctly interpreted.

In fixed tissues carried through alcohol, ether, chloroform, etc., only hemosiderin and melanin persist. The carotin is dis-

solved out with the fat; hence it must be studied in the fresh state or in frozen sections of tissues preserved in formaldehyde.

Carbon.—Occurs commonly and most abundantly in the lungs and peribronchial lymph nodes but is sometimes transplanted to the spleen and liver where its recognition occasionally is important. It must be distinguished from malarial pigment (hemozoin), from hemoglobin-formaldehyde precipitate, possibly from iron. Its distinguishing characteristics are its black color and its insolubility in concentrated sulphuric acid in which all other pigments dissolve.

Silver.—Appears brown to black; is turned black by ammonium sulphide; is removed by a mixture of ferricyanide of potassium and hyposulphite of sodium or by Weigert's ferricyanide-borax differentiating fluid. As a result of treatment therapeutically with silver nitrate silver may be deposited in the skin, intestine, spleen, kidney, etc., or in the kidney alone as the result of injection of argyrol into the pelvis of the kidney.

Iron.—Inhaled; gives iron reaction; can be removed by oxalic acid (half saturated solution for an hour or more).

Lead.—Sulphide black; gives blue line on gums in lead poisoning.

Petrifaction.—Calcification, the more common form of petrifaction, is the term applied to the infiltration of tissues with phosphate and carbonate of calcium. The salts appear microscopically as small, very refractive granules which may be mistaken for fat, or as large masses due to the fusion of granules. They are dissolved by hydrochloric or nitric acid (5 per cent. solution). If carbonate of lime is present, bubbles of carbon dioxid are set free. Phosphate of lime dissolves without effervescence. To differentiate between lime-salts and other substances soluble in hydrochloric acid use concentrated sulphuric acid to form sulphate of lime (gypsum), which appears as fine, short, radiating needles. On dissolving out the lime-salts a matrix of dead tissue or of hyaline material will usually be found left behind. As a rule, this hyaline material stains deep blue in alum-hematoxylin or red in Van Gieson's mixture.

The deposits of calcium salts themselves also stain with hematoxylin, so that it can be used to demonstrate the masses and coarser granules of them. The tissue must, however, first be freed of certain iron combinations, which are often associated with deposits of lime and also stain with hematoxylin. The following method is recommended by Roehl:

Roehl's Hematoxylin Method.—1. Fix in alcohol or formaldehyde.

2. Place sections in a half-concentrated solution of oxalic acid for fifteen to thirty minutes to remove the iron.

3. Wash thoroughly in water.

4. Stain in a 1 per cent. aqueous solution of hematoxylin (which must be neither too fresh nor too old) for five to ten minutes.

5. Differentiate in water, to which a few drops of ammonia water are added, until the section is colorless and only the lime deposits remain stained.

6. Wash in water.

7. Counterstain with safranin. Alcohol; xylol; balsam. Lime-salts deep violet; nuclei red.

Von Kossa has shown that phosphate of calcium can be demonstrated by means of nitrate of silver, which forms silver phosphate on the surface of the granules and blackens in the presence of light. It gives an exaggerated picture of the amount of lime-salts present. Klotz has shown that the nitrate of silver acting for many hours affects calcium carbonate also: the granules become coated with silver carbonate, which in sunlight gives off carbon dioxid, leaving the black silver oxid. This process can be hastened by putting the sections, after staining and thorough washing, into a dilute soluble sulphide.

Von Kossa's Silver Method.—1. Fix in alcohol, formaldehyde, or corrosive sublimate.

2. Place sections (frozen, celloidin, paraffin) in a 1 to 5 per cent. aqueous solution of nitrate of silver for thirty to sixty minutes (von Kossa), three to twelve hours (Klotz).

3. Wash thoroughly in distilled water.

4. Mount in glycerin or, after dehydration and clearing, in xylol balsam.

The lime is stained deep black. The nuclei can be counter-stained with alum carmine or safranin after the silver staining.

Another form of petrifaction is that found in gout, due to the infiltration of certain tissues with uric-acid salts, of which urate of sodium is the most common. The crystals are sol-uble with difficulty in cold water, insoluble in alcohol and ether. Therefore, to study the deposits in connection with the lesions, fix in 95 per cent. alcohol and embed in celloidin; stain sections quickly in a cold solution of alum-hematoxylin. Wash quickly in cold water and transfer to alcohol. Clear and mount in balsam.

CULTURE-MEDIA

CULTURE-MEDIA consist of various nutritive substances either liquid or solid, in or upon which bacteria will grow and multiply, and are, as a rule, contained in test-tubes ready for use.

The nutritive material in these test-tubes must be free from living bacteria—*i.e.*, "sterile"—and must be kept so until used. This is accomplished by inserting a stopper of raw cotton into the mouth of each test-tube to exclude the entrance of bacteria from without, and then subjecting the tubes and their contents to the sterilizing action of live steam for the purpose of killing any bacteria which may have gained access to the medium during its preparation.

THE PREPARATION OF GLASSWARE [1]

New glassware should be washed in a very dilute solution of nitric acid (2–5 c.c. of the commercial nitric acid to the liter of water), then thoroughly rinsed in water and allowed to drain until dry or nearly so. The object of the use of the nitric acid is to remove any free alkali which may be present.

Old glassware containing culture-media, subsequent to autoclaving and after removal of the cotton stoppers, should be boiled for from half an hour to one hour in a solution of common soda (4–6 per cent.). This treatment loosens and liquefies the old culture material, so that it may be easily removed with the aid of a brush and plenty of water.

When all the material has been removed from the glassware in this way, it should be rinsed in clean water, then in the dilute nitric acid of the strength above indicated for the new glassware, and again in clean water and allowed to drain until dry or nearly so.

[1] For routine work 16 × 125 mm. Board of Health test-tubes, 100 mm. Petri dishes, and 250 c.c. Erlenmeyer flasks are recommended.

The test-tubes and flasks thus prepared are next to be provided with stoppers of raw cotton (non-absorbent cotton), which are to be inserted into the mouths for a distance of about 3 cm., and should fit the walls smoothly. This is usually done by tearing off from a cotton roll a piece about 4 or 5 cm. square and forcing it into the mouth of the tube or flask either with a blunt rod or pair of forceps. A neater cotton plug or stopper is made as follows: starting with a piece roughly 6–7 cm. square fold in two opposite sides for 0.5–1.0 cm., roll loosely so that these squared sides form the end of the roll, bend this roll at the center, and insert into the test-tube or flask. The stoppers should not be packed in too tightly, but should be just firm enough in position to sustain easily the weight of the tube or flask when it is lifted by the projecting portion of the cotton. The stoppered tubes are then to be packed into wire baskets which fit into the hot-air sterilizer.

Petri dishes should be fitted together, and then wrapped in paper either individually or in lots of as many as are likely to be used at any one time.

Pipettes after use should be immersed for at least twenty-four hours in potassium bichromate sulphuric acid cleaning solution contained in tall glass cylinders, after which they should be washed thoroughly in warm water and dried. They should be plugged by pushing gently into the mouth end a small piece of cotton. Pipettes are usually sterilized in wrought iron or copper boxes. The safest, but at the same time most laborious, method is to wrap each pipette individually with a narrow strip of paper.[1] The paper is cut into strips about 5 cm. in width and the length is varied according to the size of pipette to be wrapped. With the long axis of the paper strip at an angle of about 30 degrees to that of the pipette, one end of the strip is folded carefully over the tip of the pipette, the small portion of the strip on the opposite side of the pipette from the main part

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[1] The best paper for this purpose is the "blue interlining paper" used commercially for wrapping up rolls of cotton. It can be purchased in small amounts usually only through some firm engaged in the manufacture of rolled cotton. Following proper dry heat sterilization the color of the paper changes from blue to bluish green.

of the strip is folded over the pipette longitudinally, the strip is wound on by rolling the pipette, and fastened by twisting the protruding end. In order to obtain a wrapping that will break easily a fairly thin paper must be used, and the overlap of the winding must be slight. With large pipettes (50–100 c.c.) it is usually advisable to leave the strip projecting at the tip and to turn this back and tie. This minimizes the chance of the wrapper being accidentally broken at the tip.

Sterilization is accomplished by heating at 150° C. for one hour. By running the oven up to 170°–180° C. one-half hour will suffice for sterilization, but there is danger of charring the cotton plugs. The object of this heating is not only to sterilize the glassware and cotton stoppers, but also to mould the stoppers to the shape of the tubes or flasks, so that they can readily be replaced when removed in the subsequent filling of the glassware with nutritive material. One should always bear in mind the fact that although the thermometer inserted through a hole in the top of the oven may register 150° C. the temperature of the bottom shelf will be considerably less. With the larger types of hot air ovens this difference may be as great as 40°–50° C.

PREPARATION OF MEDIA

Meat Extract Bouillon:

Beef extract,	5 grams;
Pepton,	10 "
Sodium chloride,	5 "
Water,	1000 c.c.

Heat with constant stirring in a kettle over a free flame until the solid ingredients are dissolved. Measure volume; and, if necessary, add water to make up for loss from evaporation. Adjust to the desired pH. Boil slowly for five minutes or place in the Arnold sterilizer for ten or fifteen minutes. Test the reaction, and readjust if necessary. Filter through paper until clear. Distribute to final containers and autoclave.

Meat Infusion Bouillon:

Minced lean beef (or veal),	500 grams;
Pepton,	10 "
Sodium chloride,	5 "
Water,	1000 c.c.

Weigh out 500 grams of beef (or veal) which has been freed from fat and passed through a meat chopper. Add 1000 c.c. of tap water, and infuse over night in an ice-box. Strain through a piece of wet cheese cloth, pressing at the end to obtain as much fluid as possible. Add the pepton and salt, and heat (not over 50° C.) with constant stirring until they are dissolved. Add water to make 1000 c.c. Adjust the reaction. Boil slowly for five minutes or place in the Arnold sterilizer for ten or fifteen minutes. Add water to make 1000 c.c. Test the reaction and, if a readjustment is necessary, repeat the heating and addition of water, and retest the reaction. Filter through cotton or paper until clear, tube and autoclave.

Another method, which results in some saving of time, is as follows: Boil the meat and water in an open kettle for thirty minutes. Filter through cotton and then proceed as in the previous method subsequent to straining the infusion through cheese cloth. In dissolving the pepton and salt no attention need be paid to the temperature of the mixture.

"Hormone" Bouillon (Huntoon):

Minced beef heart (fresh),	500 grams;
Pepton,	10 "
Gelatin,	10 "
Sodium chloride,	5 "
Water,	1000 c.c.;
Egg (whole),	One.

The heart muscle is carefully freed from fat and large blood vessels before being passed through the meat chopper. The egg should be slightly whipped. Place all the ingredients in a kettle and heat to 70° C. Add sufficient normal NaOH to render the reaction slightly alkaline to litmus and place the

mixture in an Arnold sterilizer for one hour. Separate the clot from the sides of the vessel with a glass rod and replace in the Arnold sterilizer for one and a half hours. Decant the clear bouillon from the clot, measure the volume and adjust the reaction. Autoclave and test the reaction. Readjust, if necessary. Tube and autoclave. After the first autoclaving the fine precipitate should be allowed to settle, after which the clear supernatant fluid can be readily decanted. The medium may be filtered through glass or asbestos wool, but should never be passed through cheese cloth, cotton, or filter paper. Huntoon cautions against autoclaving, and undoubtedly a somewhat better medium is obtained by fractional sterilization in the Arnold. For ordinary work it has been found that autoclaving is perfectly satisfactory.

By virtue of not being filtered certain nutritive substances are retained in this medium. Rapid growth can be obtained from freshly isolated meningococci, and the growth of such organisms as streptococci and pneumococci is much more active than in meat infusion bouillon. Huntoon states that the "growth value . . . is at least as good as the average grade serum agar."

Dextrose Bouillon.—Prepare in the same way as plain bouillon, adding 10 grams of dextrose to each liter. The dextrose should be added along with the pepton and salt.

Glycerin Bouillon.—Add 60 c.c. C.P. glycerin to each liter of meat infusion bouillon just prior to the final filtration. Sterilize by the fractional method in the Arnold.

Sugar=free Bouillon.—Prepare meat infusion broth in the usual way carrying the process through to the point of the adjustment of the reaction. Inoculate the medium with an eighteen hour bouillon culture of B. coli communis. Incubate overnight. Place in an Arnold sterilizer for one hour to kill the bacteria. Add water to original volume, and adjust to the desired reaction. Boil slowly for five minutes or heat in the Arnold for ten or fifteen minutes. Test reaction, and readjust and reheat if necessary. Filter through cotton or paper until clear.

This bouillon is used as a basis for media containing solutions of the various sugars. Weigh out samples of chemically pure sugars and add to the bouillon, after filtering, in such amounts that their concentrations are 1.0 per cent. If an indicator is desired, add 1.0 per cent. Andrade. Tube, and sterilize by the fractional method in the Arnold. In order to determine whether or not gas is formed during fermentation the culture tubes should contain a small inverted vial or test-tube (40–70 × 6–12 mm.). After sterilization it will be found that the vials or tubes are completely filled with the medium. If by chance there is a small air bubble in any of the tubes the height of the medium column should be indicated by a mark on the outside of the outer tube. If the media are autoclaved, there is great danger of splitting down the sugars in those containing polysaccharides into their simpler components.

Calcium Carbonate Bouillon.—To dextrose bouillon add 1.0 per cent. powdered calcium carbonate. Autoclave. This medium is usually placed in Erlenmeyer flasks containing about 100 c.c. It is a good plan to sterilize the calcium carbonate prior to adding it to the bouillon by heating in a hot air sterilizer.

Chocolate Broth (Williams).—Use as a basis veal or beef infusion bouillon with a reaction of pH 7.8. Heat the bouillon in the autoclave or Arnold for fifteen minutes. Immediately afterwards, while the medium is still hot (about 90° C.), add 5 per cent. sterile defibrinated blood (horse or other). Tube with aseptic precautions.

If desired the medium can be immediately filtered, tubed, and sterilized by the fractional method in the Arnold.

Pepton Solution (Dunham):

Pepton,	10 grams;
Sodium chloride,	5 "
Water,	1000 c.c.

Add the solid ingredients to the water and heat with stirring until dissolved. Filter through paper.

In using this as a basis for fermentation media, add 1.0 per cent. Andrade indicator and 1.0 per cent. of the desired sugar.

Tube, and sterilize by the fractional method in the Arnold. In order to determine gas production the culture tubes should contain small inverted vials or test-tubes, identical with those described in the method for preparing sugar-free bouillon. The reaction of the medium should be adjusted, if necessary, to about pH 7.2. This is the neutral point of the indicator and also close to the optimum reaction for the growth of the various Gram decolorized bacilli, for the differentiation of which the medium is used.

Meat Extract Agar:

Beef extract,	5 grams;
Pepton,	10 "
Sodium chloride,	5 "
Shredded agar-agar,	15 to 25 "
Water,	1000 c.c.

Place all ingredients in a kettle and autoclave at 120° C. for fifteen minutes. The amount of agar-agar added depends upon the desired firmness of the medium. Solution may be brought about by boiling over a free flame, but requires long boiling and frequent stirring. Loss from evaporation must be made up for by the addition of water, if the latter method is used. Adjust to desired reaction, and cool to 60° C. Stir in thoroughly the whites of two eggs. Autoclave for thirty minutes at 120° C. or place in an Arnold for thirty to forty-five minutes. Test the reaction; and, if readjustment is necessary, reheat. Filter, tube and autoclave.

The filtering of agar is difficult owing to the slowness of filtration which increases as the temperature drops. The temperature must never be allowed to fall below 38° C., otherwise the agar will solidify. For ordinary work a sufficiently clear medium is obtained by filtering through cotton. If filter paper is used the whole apparatus should be placed in a steam sterilizer until filtration is complete, otherwise it is necessary to reheat the unfiltered medium several times before the process is finished. Another method consists in placing the medium in a sedimenting vessel, such as a large funnel, and heating in an

Arnold sterilizer for forty-five minutes. The funnel is left in the sterilizer over night, and the next morning the medium is removed as a cast. The bottom portion, containing the sediment, is cut away and discarded. The upper portion is remelted and tubed.

Meat Infusion Agar:

Minced lean beef (or veal),	500 grams;
Pepton,	10 "
Sodium chloride,	5 "
Shredded agar-agar,	15 to 25 "
Water,	1000 c.c.

One method is as follows: Make ordinary meat infusion bouillon. Add the agar-agar and heat until dissolved. Add water to 1000 c.c., adjust the reaction and cool to 60° C. Clear with the whites of two eggs, filter, tube and autoclave.

The following method is usually preferred: Infuse the meat with 500 c.c. of water over night in the ice-box. Filter through cheese cloth, pressing out as much of the juice as possible. Add water to 500 c.c. Add the pepton and salt and heat up to 50° C., stirring until they are dissolved. In the meantime dissolve the agar-agar in 500 c.c. of water by heating in the autoclave at 120° C. for fifteen minutes. Cool to 50° C. and combine the two mixtures. Adjust to the desired reaction, add the whites of two eggs, mix thoroughly, and autoclave at 120° C. for thirty minutes or heat in the Arnold for forty-five minutes. Test the reaction; and, if readjustment is necessary, reheat. Filter, tube and autoclave. The same directions for filtering apply as for meat extract agar.

"Hormone" Agar (Huntoon).—Prepare by the same method as "hormone" bouillon, omitting the gelatin and adding in its place 16 grams of soaked shredded agar-agar.

Blood Agar.—As a base use meat infusion or "hormone" agar adjusted to a final reaction of pH 7.8. The agar is conveniently stored in 200–250 c.c. wide mouthed bottles containing 100 c.c. of the medium. Prior to use, melt the agar by immersing the bottle in a water bath at 100° C. or by placing

in the Arnold. As soon as the agar is *completely* melted, cool the bottle and its contents to about 50° C. by immersing in a water bath of suitable temperature. To each bottle add about 5.0 c.c. of sterile defibrinated blood (horse, sheep, rabbit, or human). Mix thoroughly, but in such a way that bubbles are not formed. Pour into sterile Petri dishes (eight or nine for each 100 c.c.), or transfer to sterile test-tubes and slant.

Chocolate Agar (Williams).—Use a base veal infusion agar containing 5 per cent. glycerin. The agar should be sterilized by the fractional method in the Arnold and should have a final reaction of pH 7.8. Immediately after the final sterilization, while the medium is still hot (about 90° C.), add 5 per cent. sterile defibrinated blood (horse or other). Tube and slant or plate.

Liver Agar.—To 500 grams fresh finely ground pig's liver add 500 c.c. of water and infuse over night in the ice-box. Heat until complete coagulation takes place and strain through cheese cloth. Dissolve 17 grams of agar-agar in 500 c.c. of water by heating in the Arnold or autoclave. Add to infusion and make up volume to 1000 c.c. Add 10 grams of pepton and 5 grams of sodium chloride, stirring until dissolved. Adjust reaction to pH 7.5. Heat in the Arnold for fifteen minutes. Filter through cotton, tube, and sterilize by the fractional method in the Arnold.

If a particularly clear medium is desired, cool to 50° C. following adjustment of the reaction, and stir in well the whites of two eggs. Heat in the Arnold for thirty to forty-five minutes instead of fifteen.

Sodium Oleate Agar (Avery).—Prepare a sterile suspension of human or rabbit red blood corpuscles by defibrinating the blood, centrifuging, pipetting away the serum, and adding plain bouillon up to the original volume. To 96 c.c. meat infusion or "hormone" agar heated to 80° to 90° C. add 1.0 c.c. of the corpuscle suspension and 5 c.c. of a sterile 2 per cent. neutral aqueous solution of sodium oleate (Kahlbaum).

Dextrose Agar.—Prepare in the same way as plain agar, adding 10 grams of dextrose to each liter of medium. The dextrose may be added along with the pepton and salt.

Glycerin Agar.—Add 60 c.c. C. P. glycerin to each liter of meat infusion agar just prior to the final titration. Sterilize by the fractional method in the Arnold.

Meat Extract Gelatin:

Beef extract,	5 grams;
Pepton,	10 "
Sodium chloride,	5 "
Finest French sheet gelatin,	140 to 180 "
Water,	1000 c.c.

Dissolve the extract, pepton and salt in the water by heating in the Arnold. Add the gelatin and place the mixture again in the Arnold until the gelatin is dissolved. Measure the volume and adjust the reaction. Cool to 50° C. and stir in thoroughly the whites of two eggs. Place in the Arnold for forty-five minutes. Test the reaction, and if readjustment is necessary, heat in the Arnold for thirty minutes. Filter, tube, and sterilize by the fractional method in the Arnold. Gelatin should *never* be autoclaved and all unnecessary heating is to be avoided.

Meat Infusion Gelatin.—Prepared as above except that the pepton, salt and gelatin are added to the meat infusion which has been filtered through cheese cloth and made to volume with water.

Blood Serum (Löffler):

Dextrose meat infusion bouillon,	1 part;
Beef blood serum,	3 parts.

The blood serum is collected at a slaughter house in tall glass jars holding 3 liters or more. Preferably the jars should be sterile, although this is not absolutely essential. Blood obtained by the Jewish method of slaughter, *i.e.*, by severing the carotid artery, is the best for the purpose, as it clots more readily. As the blood runs from the vessels of the animal it is received in the jar and immediately placed in the ice-box for twenty-four to forty-eight hours. All unnecessary agitation of the fresh blood should be avoided as this interferes with its proper clotting. It is well to inspect the blood after a few hours, and gently loosen with a glass rod any adhesions which the clot

may have formed to the wall of the jar. After twenty-four hours the serum is removed with a pipette. If the clot is in good condition more serum may be obtained after another twenty-four hours. The presence of a few red blood corpuscles in the serum is of little importance. Hog serum may be used in place of beef serum.

Mix the serum and bouillon, and tube the mixture. Slant in an inspissator, heat slowly up to 90° C. (this should take thirty to forty-five minutes) and maintain this temperature for two and a half to three hours. A discarded incubator of the water bath type can be used for this purpose. Do not pack the slanted tubes too tightly. Unless some air spaces are left for circulation the innermost tubes will not be sufficiently coagulated. After coagulation, pack the slants upright in wire baskets and autoclave for one hour at 120° C.

The above is a modification of Löffler's original method. The ingredients are the same, but the method of coagulating is greatly simplified. The resulting medium is opaque and firm, whereas the original medium is fairly transparent and relatively soft. For throat cultures and other routine work the modified medium is perfectly satisfactory, and the saving in time and effort warrants its use.

If the fairly transparent medium is desired, proceed as follows: Collect the serum with aseptic precautions. Mix as above. Inspissate for one hour on five successive days at 68° to 70° C. After the fifth sterilization solidify the tubes in the form of "slants" by slowly raising the temperature to 80° C. Maintain this temperature for several hours.

Serum Water Media (Hiss).—To one part of clear serum (beef, horse, rabbit, or human) add two or three parts of distilled water. Heat in the Arnold for fifteen minutes. Add a sufficient amount of aqueous litmus solution to impart a deep blue color to the mixture. To different portions add 1.0 per cent. of the desired sugars.[1] Sterilize by the fractional method in the Arnold.

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[1] In preparing medium containing inulin, dissolve the inulin in the water to be used for diluting the serum and sterilize the solution in the autoclave at 120° C. for fifteen minutes. This is necessary to kill the spores which are usually present.

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Another convenient method consists of preparing stock serum water medium containing indicator and stock 10 per cent. solutions of the various sugars. All are sterilized by the fractional method. The final medium is obtained by mixing in a sterile flask nine parts of medium and one part of the desired sugar and pipetting the mixture into sterile test-tubes.

Potato Medium.—Wash and pare several large potatoes—preferably old ones—and then cut out solid cylinders with a cork-borer or apple-corer. These cylinders should be about 5 cm. long and of a suitable diameter to fit into ordinary culture tubes. Halve the cylinders by cutting longitudinally in an oblique direction. Wash the pieces in running water over-night or soak them in a 1.0 per cent. solution of sodium carbonate for half an hour to neutralize the normal acidity of the vegetable. Place in tubes, adding a small amount of water. Sterilize by the fractional method. If desired, a small piece of glass rod may be placed in the bottom of the tube to elevate the potato above the water (Fig. 14).
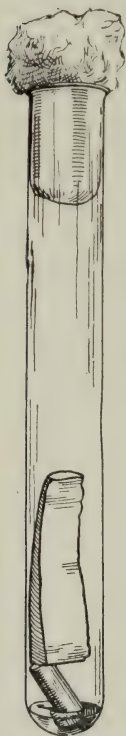
Glycerin Potato Medium.—Before tubing immerse the pieces of potato in a 10 to 20 per cent. solution of glycerin for several hours. Add to the culture tubes a small amount of 10 per cent. glycerin instead of water. Otherwise prepare the same as plain potato medium.

Litmus Milk.—To *fresh* fat-free milk (*pH* 7.0 *or higher*) add sufficient aqueous litmus solution to impart a distinct blue color. Tube, and sterilize by the fractional method in the Arnold.

FIG. 14.—Potato-culture.

Egg Medium (Dorsett).—Break fresh eggs under aseptic precautions into a wide-mouthed sterile flask. The shells should be previously sterilized by immersing the eggs for ten minutes in 70 per cent. alcohol. Mix the whites and yolks by shaking. To every four eggs add 25 c.c. sterile water.

Strain through sterile gauze. Transfer aseptically to sterile test-tubes. Slant and harden slowly in an inspissator at 73° to 76° C. for four or five hours on three successive days. On the first two days hold the temperature at 73° C. and on the last day raise to 76° C. Just before inoculating add 3–4 drops of sterile water to each slant in order to supply the moisture required for the satisfactory development of the tubercle bacillus.

Russell Double Sugar Agar.—To meat extract or infusion agar of the proper reaction add an indicator and 1.0 per cent. lactose and 0.1 per cent. dextrose dissolved in a small volume of water. Tube, sterilize fractionally in the Arnold and slant.

As an indicator Russell used an aqueous solution of litmus, and the reaction of the medium was adjusted to neutrality with this indicator. Andrade indicator (1.0 per cent.) gives more satisfactory results providing the reaction of the medium is correct ($\pm$pH 7.2).

Each lot of medium should be standardized by culturing proved cultures of the typhoid, paratyphoid and dysentery groups.

Endo Medium.—As a basis use meat extract or infusion agar (2.5–3.0 per cent.) of the proper reaction. Store in flasks or bottles containing 100 c.c. of the medium base. Before use, add 1.0 gram (1.0 per cent.) of lactose and place the flask or bottle in the Arnold for thirty minutes. This heating melts the agar and the lactose is dissolved and sterilized. Add 0.25 c.c (0.25 per cent.) of a filtered saturated alcoholic solution of basic fuchsin and a sufficient amount of a freshly prepared 10 per cent. solution of sodium bisulphite in sterile water. Sterilize in the Arnold for twenty minutes and pour into sterile Petri dishes. Allow the plates to harden with the covers off protected from the dust in a drawer or cupboard.

The final reaction of the medium should be pH 7.8–8.0. If the reaction of the agar is in the neighborhood of pH 8.5, the addition of the sodium bisulphite plus sterilization will reduce the alkalinity to the desired point. The amount of sodium bisulphite solution to be added varies greatly (0.5–2.0

per cent.) and is most likely dependent upon the purity of the sodium bisulphite. It should be added to the hot mixture until the brilliant red of the fuchsin is transformed to a pale rose. When cold, the medium should be a very faint pink.

Kendall's modification consists of adding to melted lactose (1.0 per cent.) agar 1.0 c.c. of a decolorized fuchsin solution prepared by adding 1.0 c.c. of a saturated alcoholic solution of basic fuchsin to 10 c.c. of a freshly prepared 10 per cent. aqueous solution of sodium bisulphite. The final concentration of the decolorized fuchsin solution in the medium is approximately one-third as compared with the concentration in the method given above.

On this medium typhoid, paratyphoid and dysentery bacilli grow as rather small colorless colonies. The colonies of organisms belonging to the colon group are generally larger and are pink in color, and the surrounding medium is usually colored red.

Brilliant Green Agar (**Krumwiede**).—Prepare *clear* stock agar containing 0.3 per cent. beef extract and 1.5 per cent. agaragar. Adjust reaction to about pH 8.0. Place in 100 c.c. amounts in bottles and autoclave. For use, melt and to each 100 c.c. add 1.0 c.c. Andrade indicator, sufficient normal HCl to neutralize, 5 c.c. of a sterile (Arnoldized) aqueous solution containing 20 per cent. lactose and 2 per cent. dextrose and a sufficient amount of a 0.1 per cent. aqueous solution of brilliant green. Mix and from each bottle pour six plates. Allow the medium to harden with the covers off in a protected place. Use porous earthenware covers, as it is essential that the medium surface be dry.

The amount of normal HCl to be added can be determined for each lot of agar by adding Andrade indicator to one bottle of melted agar, distributing the medium in 10 c.c. amounts to a series of clean test-tubes, adding 0.1 normal HCl in varying amounts and cooling. Agar neutral to the indicator is red when hot and fades on cooling. The amount of 0.1 normal HCl added to the 10 c.c. sample is the amount of normal HCl to be added to one 100 c.c. bottle.

The 0.1 per cent. brilliant green solution is made by dissolving 0.1 gram of the dye in 100 c.c. of distilled water contained in a clean 100 c.c. volumetric flask.   Ordinarily 0.3 c.c. and 0.2 c.c. of this solution (corresponding respectively to 1 : 330,000 and 1 : 500,000 dilutions of the solid dye) are added to separate bottles of the medium.   However, *each new dye solution and each new batch of agar varies so that it is necessary to restandardize for each.*   The dye solution keeps for about a month.

Standardization is best accomplished by plating out one or more positive typhoid stools.   Such a stool may be simulated by inoculating normal stool suspensions with an eighteen hour typhoid broth culture (1 loopful to 10 c.c. of suspension). Suspensions should be of such density that discreet colonies are obtained.   Inoculate on media containing 0.5, 0.3 and 0.2 c.c. of the dye solution.   From the results obtained choose two dilutions: (1) The largest amount of dye which does not influence the size or number of typhoid colonies but limits the growth of the normal fecal flora, and (2) a larger amount which limits the size and number of the typhoid colonies and restrains to a high degree the fecal flora.   As mentioned above, the dilutions are most commonly 1 : 500,000 and 1 : 330,000, corresponding to 0.2 and 0.3 c.c. of the 0.1 per cent. dye solution.

Typhoid colonies viewed against a dark background with oblique light have a characteristic snowflake appearance, and with a hand lens under the same conditions they appear as bits of coarse wool fabric.   They may be slightly tinted due to acid fermentation of the dextrose.   Occasionally, on a certain batch of medium, very large atypical typhoid colonies develop. Paratyphoid B colonies are usually larger and thicker, as the dye exerts but slight inhibitory action.   Paratyphoid A colonies resemble those of typhoid, but usually are much thinner.   The growth of organisms belonging to the dysentery and colon (with the exception of B. lactis aërogenes) groups is generally almost completely inhibited.   The colonies of the fecal types which do develop are usually pink in color—lactose fermenting.   In appearance they can be confused but rarely with typhoid colonies.

FILTERING OF MEDIA

Filter Paper.—Media which contain but little or no coagula may be filtered by passing through filter paper. The soft, rapidly filtering grade should be used. The process may be hastened by folding the papers so as to form flutes, and by filtering through several funnels simultaneously. It is advisable to use two thicknesses of paper in each funnel.

Cotton.—Pace a ball of "excelsior" 2 to 3 cm. in diameter in the apex of the funnel. Take a piece of roll absorbent cotton 10 to 12 cm. square and split it to form two thin squares of the same size. Place one square above the other so that the fibers run at right angles with the fibers of the other. Press the square firmly down on the "excelsior," moisten the cotton with water and smooth the edges against the glass wall. Pour in the medium very slowly, directing it to the center of the cotton by means of a glass rod. After the funnel is filled to the top, keep it fairly well filled until the process is complete. The first portion filtering through is usually quite cloudy and should be refiltered. Agar, gelatin, and media containing appreciable amounts of coagula are best filtered in this way. In filtering agar or gelatin it is advisable to place some sort of a cover over the top of the funnel in order to conserve the heat.

ADJUSTMENT OF MEDIUM REACTION

Titration Method.—Until recently the titration of a medium has consisted in determining the amount of acid or alkali required to bring the medium to neutrality using phenolphthalein as an indicator. This is accomplished as follows: Remove 5 c.c. of the medium to a porcelain evaporating dish, add 45 c.c. distilled water and boil for three minutes to expel the free CO_2. Add 1.0 c.c. of a 0.5 per cent. solution of phenolphthalein in 50 per cent. alcohol. If the mixture is colorless, the reaction is on the acid side of neutral, and if pink or red, is on the alkaline side. From a burette add $\frac{N}{20}$ sodium hydroxide, or $\frac{N}{20}$ hydrochloric acid, with constant stirring until a distinct

pink color persists, or until the solution becomes colorless. From the amount of $\frac{N}{20}$ alkali, or $\frac{N}{20}$ acid, used to neutralize 5 c.c. calculate the amount of normal alkali, or normal acid, required to neutralize the total quantity of medium.

The reaction of a medium has been but rarely adjusted to neutral. The majority of organisms grow best in media with a reaction slightly on the acid side of neutrality. For some the optimum reaction is one that would require the addition of 0.5 per cent. normal sodium hydroxide to render the medium neutral to phenolphthalein. Such a reaction is designated "+0.5 per cent.," and is created in a medium by adding the calculated amount of normal sodium hydroxide necessary to neutralize, minus 5.0 c.c. (0.5 per cent.) per liter. Conversely, a reaction of "−0.5 per cent." means that 0.5 per cent. of normal hydrochloric acid would have to be added to render the medium neutral. A reaction of "+0.5 per cent." is most practical for the cultivation of the majority of pathogenic bacteria. The Gram decolorized bacilli and other organisms recovered from the gastro-intestinal tract grow best in media with a more acid reaction (+1.0 per cent.).

Hydrogen Ion Concentration (Colorimetric) Method.—*Acids*. In order to understand what is to follow it will be necessary to consider very briefly a few of the properties of acids. When any acid is dissolved in water a certain portion of the acid is split up or dissociated into hydrogen ions and anions (chlorine ions in the case of hydrochloric acid). This dissociation is constant for a given acid, but varies greatly with different acids. With hydrochloric, nitric, and sulphuric acids the dissociation is practically complete, and nearly all the hydrogen is present as hydrogen ions. With acetic and lactic acids the degree of dissociation is slight and nearly all the hydrogen is present in combination with the anion as undissociated acid. Consequently, although the same amount of alkali is required to neutralize solutions of the same normality of all acids, the concentration of hydrogen ions varies greatly, being high with the highly dissociated acids and low with the

weakly dissociated acids. In other words, titratable acidity and hydrogen ion concentration are not identical. For example, if two solutions, one of $\frac{N}{10}$ hydrochloric acid and one of $\frac{N}{10}$ acetic acid, were adjusted to a reaction of "$+1.0$ per cent." by the old titration method, the hydrogen ion concentration of the former would be much greater than that of the latter. Recent investigators have shown that the vital factor of reaction, as regards bacterial growth, is hydrogen ion concentration rather than titratable acidity.

Buffers.—Another factor, which is more or less dependent upon the foregoing considerations, is the presence of buffer substances in media. A buffer is any substance which by its presence in a solution tends to limit a change of reaction on the addition of acid (or base). For example, if 10 c.c. $\frac{N}{10}$ hydrochloric acid are added to 50 c.c. of a mixture of equal parts $\frac{N}{10}$ K_2HPO_4 and KH_2PO_4, the hydrogen ion concentration is changed from pH 6.8 to pH 6.5. The acid unites with the K_2HPO_4 to form KH_2PO_4 and KCl. In this way the hydrogen ions are used to form an acid salt (KH_2PO_4), which acts as a very weakly dissociated acid ($H\cdot KHPO_4$). The majority of hydrogen ions are held in combination with the anions ($KHPO_4$) in the form of undissociated $H\cdot KHPO_4$, and the actual increase in hydrogen ion concentration is slight. On the other hand, if the same amount of acid were added to 50 c.c. of freshly distilled water, the increase in hydrogen ion concentration would be great (pH 7.0 to pH 1.8), for all of the hydrogen would be present as dissociated hydrogen ions. Phosphates are examples of buffers, and a solution containing phosphates is said to be buffered. The higher the concentration of buffers in a solution, the more the opposition to reaction change. Proteins, protein split products (such as pepton), and carbonates as well as phosphates act as buffers, and are present in varying amounts in different kinds of media. The inaccuracy of adjusting the reaction by the titration method is apparent if we con-

sider the end results of a "+1.0 per cent." adjustment on the one hand with well buffered and on the other with poorly buffered media. It follows that the reaction of media should be adjusted by determining the proper amount of alkali (or acid) to be added to create the desired hydrogen ion concentration.

Nomenclature.—Considerable confusion exists in the mode of expressing hydrogen ion concentration. For example, all the following expressions refer to the same hydrogen ion concentration:

$$\text{pH } 7.3, \ [H^+] = 10^{-7.3}, \ [H^+] = 50 \times 10^{-9},$$
$$[H^+] = 5 \times 10^{-8}, \text{ and } [H^+] = 0.5 \times 10^{-7}.$$

In order to correlate these various modes it will be necessary to consider briefly certain fundamental facts. The hydrogen ion concentration, $[H^+]$, of any solution is a figure expressing the concentration of the hydrogen ions in the solution as compared with those in a theoretical solution which is normal with respect to hydrogen ions at standard conditions of temperature and pressure. Assuming that an ordinary normal solution of hydrochloric acid represents this theoretical solution, then the $[H^+]$ of dilutions of this normal solution may be expressed as follows:

$$0.1N, \quad [H^+] = \frac{1}{10} \ = \frac{1}{10^1} = 10^{-1} = 10^{1-2} =$$
$$10 \times 10^{-2} = 100 \times 10^{-3} = \text{pH } 1.0$$

$$0.05N, \quad [H^+] = \frac{1}{20} \ = \frac{1}{10^{1.3}} = 10^{-1.3} = 10^{0.7-2.0} =$$
$$5 \times 10^{-2} = \ 50 \times 10^{-3} = \text{pH } 1.3$$

$$0.01N, \quad [H^+] = \frac{1}{100} \ = \frac{1}{10^2} = 10^{-2} =$$
$$1 \times 10^{-2} = \ 10 \times 10^{-3} = \text{pH } 2.0$$

$$0.005N, \quad [H^+] = \frac{1}{200} \ = \frac{1}{10^{2.3}} = 10^{-2.3} = 10^{-0.3-2.0} =$$
$$0.5 \times 10^{-2} = \ 5 \times 10^{-3} = \text{pH } 2.3$$

$$0.001N, \quad [H^+] = \frac{1}{1000} = \frac{1}{10^3} = 10^{-3} = 10^{-1-2} =$$
$$0.1 \times 10^{-2} = \ 1 \times 10^{-3} = \text{pH } 3.0$$

The method most generally employed at present to express
$[H^+]$ is that devised by Sørensen, namely:

$$pH = -\log [H^+].$$

For example, with 0.05N hydrochloric acid $[H^+] = 10^{-1.3}$;
then, $\log [H^+] = -1.3$ and $-\log [H^+] = -(-1.3) = 1.3 =$
pH. Although Sørensen's method simplifies matters a great
deal, it must be acknowledged that the relative strengths of a
series of dilutions are more apparent when the $[H^+]$ are expressed
as multiples of a common factor—for example, 10^{-3} in the fore-
going series of dilutions.

It has been found that water dissociates so that the product
of the hydrogen and hydroxyl ion concentrations is always
equal to 10^{-14}. This holds true for all aqueous solutions.
It follows that in a neutral solution the $[H^+]$ and $[OH^-]$ must
both equal $10^{-7}(10^{-7} \times 10^{-7} = 10^{-14})$. If the $[H^+]$ is greater
than 10^{-7} (less than pH 7.0), the hydrogen ions are in excess and
the solution is acid. If the $[H^+]$ is less than 10^{-7} (greater
than pH 7.0), the hydroxyl ions are in excess and the solution
is alkaline.

Measurement of Hydrogen Ion Concentration.—By far the
most accurate means of determining hydrogen ion concentra-
tion is by the electrometric or "gas chain" method. For
ordinary bacteriological work the method is not practical,
because of the equipment and time required. The colorimetric
method is accurate within ± 0.1 pH, requires but little equip-
ment and can be easily and quickly carried out.

The colorimetric method is dependent upon the fact that
certain dyes, "indicators," undergo definite color changes in
solutions of varying hydrogen ion concentration. These color
changes are most rapid per unit change of reaction at a certain
hydrogen ion concentration, which varies with different indi-
cators. The color changes diminish so quickly that they are of
no practical importance when ± 1.0 pH from the point of maxi-
mum change. Clark and Lubs[1] recommend the following list
of indicators, which cover a range from pH 1.2 to pH 9.8:

[1] Clark and Lubs, *J. Bact.*, 1917, ii, 1, 109 and 191.

Thymol blue (acid range),   1.2–2.8
Brom phenol blue,   3.0–4.6
Methyl red,   4.4–6.0
Brom cresol purple,   5.2–6.8
Brom thymol blue,   6.0–7.6
Phenol red,   6.8–8.4
Thymol blue (alkaline range),   8.0–9.6
Cresol phthalein,   8.2–9.8

In order to make colorimetric comparisons it is necessary to prepare standard buffer solutions. The reaction of these solutions should be checked by electrometric measurements, whenever possible. However, if great care is taken in their preparation (us'ng redistilled water, recrystallized salts, carbonate-free NaOH, etc.), this check method may be omitted with a fair degree of security. Clark and Lubs[1] have designed a series of standard sets which cover a range from pH 1.0 to pH 10.0. In their paper will be found careful directions for preparation of these solutions. One of these sets and a portion of another are given below. They cover the range which is important in general bacteriological work. The solutions should be kept in alkali-free glass bottles or flasks, preferably with 10 c.c. pipettes thrust through the stoppers.

For pH 5.8, To 50 c.c. $M/5$ KH_2PO_4 add 3.72 c.c. $M/5$ NaOH, and dilute to 200 c.c.
"   " 6.0 "   "   "   "   "   "   5.70 "   "   "   "   "   "   "   "
"   " 6.2 "   "   "   "   "   "   8.60 "   "   "   "   "   "   "   "
"   " 6.4 "   "   "   "   "   " 12.60 "   "   "   "   "   "   "   "
"   " 6.6 "   "   "   "   "   " 17.80 "   "   "   "   "   "   "   "
"   " 6.8 "   "   "   "   "   " 23.65 "   "   "   "   "   "   "   "
"   " 7.0 "   "   "   "   "   " 29.63 "   "   "   "   "   "   "   "
"   " 7.2 "   "   "   "   "   " 35.00 "   "   "   "   "   "   "   "
"   " 7.4 "   "   "   "   "   " 39.50 "   "   "   "   "   "   "   "
"   " 7.6 "   "   "   "   "   " 42.80 "   "   "   "   "   "   "   "
"   " 7.8 "   "   "   "   "   " 45.20 "   "   "   "   "   "   "   "
"   " 8.0 "   "   "   "   "   " 46.80 "   "   "   "   "   "   "   "
For pH 7.8, To 50 c.c. $M/5$ $(H_3BO_3 + KCl)$ "   2.61 "   "   "   "   "   "   "   "
"   " 8.0 "   "   "   "   "   "   "   3.97 "   "   "   "   "   "   "   "
"   " 8.2 "   "   "   "   "   "   "   5.90 "   "   "   "   "   "   "   "
"   " 8.4 "   "   "   "   "   "   "   8.50 "   "   "   "   "   "   "   "
"   " 8.6 "   "   "   "   "   "   " 12.00 "   "   "   "   "   "   "   "
"   " 8.8 "   "   "   "   "   "   " 16.30 "   "   "   "   "   "   "   "
"   " 9.0 "   "   "   "   "   "   " 21.30 "   "   "   "   "   "   "   "

[1] Clark and Lubs, *J. Biol. Chem.*, 1916, xxv, 479

To prepare the colorimetric scale pipette 10 c.c. amounts of the various standard solutions to test-tubes of *uniform* diameter. The tubes should be thoroughly clean and, prior to filling, should be rinsed with redistilled water. Add 5 drops of the proper indicator solution. The indicators to be used with the solutions given above are brom thymol blue (6.0–7.6) phenol red (6.8–8.4) and thymol blue (8.0–9.6). The concentrated indicator solutions (0.4 per cent.) are prepared by grinding 0.1 gram of the dry powder until dissolved in an agate mortar with 3.2 c.c., 5.7 c.c. and 4.3 c.c. $\frac{N}{20}$ NaOH respectively and adding distilled water to 25 c.c. These concentrated solutions should be diluted so that the final concentrations are 0.04, 0.02 or 0.03, and 0.04 per cent. respectively. For accurate work the colorimetric standards should be made up fresh every day. In adjusting the reaction of media fresh standards should be made at least every two weeks, and in the meantime the tubes should be kept well stoppered and in the dark.

To determine the pH of a colorless clear solution, pipette 10 c.c. into a test-tube of the same diameter as those used for the colorimetric scale, add 5 drops of indicator solution and compare with the standard tubes. A reading within ±0.05 pH can easily be made. In measuring and adjusting the reaction of media there is usually some color and a slight turbidity. In such instances it is necessary to use a comparator block, whereby the solution plus indicator is compared with standard tubes in front of which are placed tubes containing only the solution. Such a block should have three pair of holes 10–12 cm. deep and of sufficient diameter to admit the test-tubes used for testing. Three smaller holes should be bored through from the front to the back of the block so that they pass through each pair of larger holes about 3 cm. above the bottoms of the latter. The block should be painted a dull black. By placing the test-tubes as indicated in the following diagram, reading through the horizontal windows, and changing the standard tubes as required, accurate determinations can easily be made:
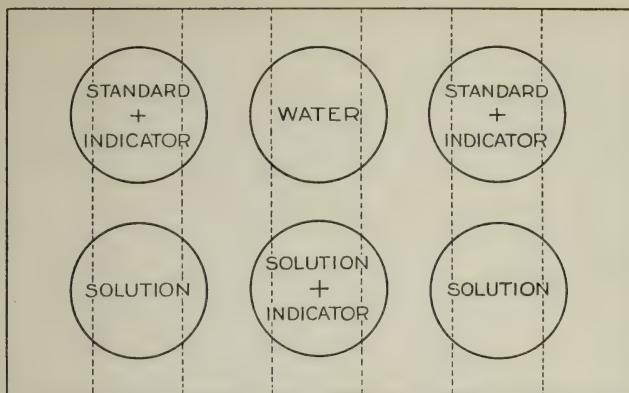
In adjusting media, pipette 5 c.c. into a test-tube and add an equal amount of distilled water. This dilution reduces the color and turbidity and does not appreciably alter the reaction. If desirable, even a higher dilution (2 c.c. + 8 c.c.) is permissible. The temperature of the solution theoretically should be that at which the medium is to be used (37° C.). Within rather wide limits this is not of practical importance. However, the temperature of the solution certainly should not be greater than 50° C. In adjusting agar the temperature should be sufficiently high (above 40° C.) to prevent solidification. Add 5 drops of the proper indicator, and then from a burette run in $\frac{N}{20}$ NaOH (or HCl) until the color corresponding to the desired reaction is obtained. From the amount of $\frac{N}{20}$ NaOH (or HCl) required to adjust 5 c.c. of the medium calculate the amount of $\frac{N}{1}$ or 5N NaOH (or HCl) required to adjust the total batch.

For general bacteriological work the final reaction should be in the neighborhood of pH 7.6–7.8. After primary sterilization following adjustment the reaction is usually 0.2–0.3 pH lower than the adjusted reaction. Consequently media, in which a final reaction of pH 7.6–7.8 is desired, should be adjusted to pH 7.9–8.1. Certain organisms, including the Gram decolorized bacilli isolated from the gastro-intestinal tract,

grow best in practically neutral media (pH 6.8–7.2). Such media should be adjusted to pH 7.1–7.5. This drop in pH is not an absolutely constant factor, and the reaction of each lot of medium should be tested subsequent to primary heating or prior to final sterilization. Readjustment should be made, if necessary.

A reaction of "+0.5 per cent.," as measured by the titration method, is usually equivalent to about pH 7.5 and one of "+1.0 per cent." to pH 6.8–7.0. It must be borne in mind that, whereas media were usually adjusted to a point on the acid side of neutrality to phenolphthalein by the titration method, media adjusted according to hydrogen ion concentration are generally neutral or slightly alkaline.

In the absence of standard buffer solutions media can be adjusted roughly to pH 7.8 by the following method. Add $\frac{N}{1}$ NaOH to the total batch of medium until the first faint pink color is noticed on mixing a drop of the medium with a drop of 0.5 per cent. phenolphthalein in 50 per cent. alcohol. The mixing should be done on the bottom of a white enamel or agate-ware tray. The change in color can be detected most readily by comparison with a drop of medium plus a drop of water. Using this method routinely, media have been obtained in which, with a few exceptions, pneumococci have thrived.

INDICATORS FOR MEDIA

Litmus.—Dissolve 5 per cent. cube litmus or azolitmin in water with the aid of heat. Filter and sterilize.

Andrade Indicator:

Aqueous acid fuchsin (0.5 per cent.),	100 c.c;
Normal sodium hydroxide,	16 "

The mixture should be yellow. If the red color persists after two or three hours, add 1.0 c.c. more of the sodium hydroxide.

The ideal final reaction of media containing Anrade indicator is pH 7.2. Media of this reaction containing 1.0 per cent. of the indicator are red when hot and faintly yellow when cold.

TUBING AND BOTTLING OF MEDIA

The filling of the test-tubes with the fluid culture-media described in this section is best effected by means of a funnel of a capacity of about a liter. In this the fluid medium is placed, and by means of a pinch-cock the requisite quantity of medium is run into each test-tube. In running the medium

Fig. 15.—Method of filling test-tubes with culture-medium (Warren).

into the test-tubes the left hand holds the test-tube while the right hand removes the cotton stopper and manipulates the pinch-cock (Fig. 15). Care should be exercised not to allow any of the medium to come in contact with the neck of the test-tube, for it will make the cotton stopper stick to the walls of the tube. To avoid this, the delivery-tube of the apparatus should be inserted some distance into the test-tube in filling.

The quantity of culture-medium run into each test-tube varies according to the form of culture desired and the character of the medium. In the case of liquid media and solid media designed to be used in the form of "stab" cultures the tubes should be filled to a depth of 5–10 cm. (Fig. 16, *a*). For "slant" cultures of solid media a depth of about 3 cm. is sufficient, or enough to give a slanting surface from the bottom of the tube to about halfway up the side wall (Fig. 16, *b*).

Small Erlenmeyer flasks are often used for bouillon cultures. These should be of about 125 c.c. capacity, and should be filled to a depth of about 1 cm. with the medium. The necks are provided with cotton stoppers, and the whole sterilized and treated as test-tube cultures.

Agar for plate cultures is conveniently stored in 100 c.c. amounts in wide-mouthed bottles having a capacity of 240–300 c.c. Stock meat infusion or bouillon may be kept in liter bottles having a square or oblong cross-section, thus economizing on space.

FIG. 16.—"Stab" culture (*a*); "slant" culture (*b*).

With flasks or bottles of media, which are to be kept for some time, it is advisable to protect the stoppers from dust by covering them before final sterilization with caps of parchment paper firmly tied down around the neck.

STERILIZATION OF MEDIA

In general all media should be placed in the incubator for twenty-four to forty-eight hours subsequent to sterilization in order to insure complete sterility.

Autoclave.—Autoclaving for fifteen to thirty minutes at 120° C. (15 lbs. pressure) is sufficient for the sterilization of all culture-media. The autoclave should be provided with a pet-cock or valve leading from a dependent part of the steam chamber, and this should be left open until steam escapes freely. If an appreciable amount of air is left in the chamber, and the instrument is controlled by a pressure gauge, there is great danger of improper sterilization due to the lack of sufficient heat. If possible, the sterilization process should be controlled with a thermometer rather than a pressure gauge.

Arnold Sterilizer.—All media containing sugars, glycerin, gelatin, and animal serum (with the exception of Löffler's blood serum) should be sterilized by exposing to live steam at 100° C. for twenty to thirty minutes on three consecutive days. In the meantime all such media should be kept at incubator or room temperature in order to allow for the development of spores to vegetative forms.

Inspissator.—Media containing serum or albuminous material may be sterilized in an inspissator without coagulation by exposing to a temperature of 60° to 73° C. for one hour on five or six consecutive days.

Unglazed Porcelain Candles.—These are used chiefly to render cultures free from bacteria. They consist of hollow candles of unglazed porcelain, closed at one end and at the other cemented to a metal base with a nipple attached which connects with the inside of the candle. This nipple is thrust through a one-hole rubber stopper which fits into the neck of an ordinary glass filtering flask. A glass mantle surrounds the candle, and the fluid to be filtered is poured into the space between the mantle and the candle. Suction is applied to the side arm of the filtering flask and the fluid is drawn through the pores of the candle, eventually dropping down from the nipple into the flask. The pores of the candle are so small that the bacteria are held back.

The candles should be sterilized in the hot air sterilizer or autoclave. All the rest of the apparatus should be autoclaved or sterilized by boiling. Following use, the candles should

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be sterilized by placing in a solution of a non-coagulating disinfectant. Then scrub carefully with a fine brush, and wash by running through 0.5 per cent. potassium permanganate solution, 5 per cent. sodium bisulphite solution and finally plenty of distilled water. The washing is accomplished preferably by running the solutions through the candle in the opposite direction from that of the actual filtering process.

CULTURE METHODS

THE bacteriological examination of material obtained from the individual during life or at autopsies should determine whether bacteria are present or not, and if present their species and comparative number. At autopsies the examination should also determine the extent of the distribution of any infecting bacteria throughout the principal internal organs.

This is accomplished chiefly by means of two methods of examination—viz., the direct examination with the microscope of smear preparations, and the results of cultures made from the tissues. Both of these methods should be employed together, but the culture method is perhaps the most important. A third but less frequent method is the inoculation of animals with pieces of tissue or material taken from the body.

Methods of Collecting Material.—In the bacteriological examination of pathological material obtained from the individual during life, it is of obvious importance that the material be protected from the invasion of bacteria from without, and that in its collection every object with which it comes in contact be free from living bacteria.

To fulfil these requirements the material may be conveniently collected in any of the following ways:

1. It may be obtained directly from the individual by means of the sterilized platinum wire, and smear preparations, cultures, and, if necessary, animal inoculations, made at once.

2. Since a very small quantity of the material usually suffices for the purposes of examination, it may often be very conveniently collected and brought to the laboratory on the so-called "swabs," where it can be subjected to the various manipulations at leisure.

The "swab" consists of a piece of rather stiff wire about 6 inches long or a piece of wood about 2 mm. in diameter and of such length that it projects 0.5–1.0 cm. beyond the mouth of the test-tube,[1] on one end of which is firmly twisted a pledget of absorbent cotton, so that the end of the wire is well covered. This is placed, cotton end first, in a test-tube, which is then provided with a cotton stopper (Fig. 17), and the whole sterilized in a hot-air sterilizer by heating to 150° to 180° C. for about half an hour. A large number of "swabs" in test-tubes may be kept on hand sterilized and ready for use.

When it is desired to secure material for bacteriological examination on a "swab," the cotton stopper is removed, the swab taken out, and the cotton end brought in contact with the pus or exudate in such a manner that some adheres to the cotton. The swab is then immediately replaced in the test-tube, the cotton stopper returned to its place, and the whole then carried to the laboratory.

In these manipulations care should be taken to avoid touching with the swab anything but the material which it is desired to examine, otherwise the material may be contaminated with other bacteria than those originally present in it.

By means of swabs material for examination from pus or exudates may be secured and brought to the laboratory in most instances. They are especially useful in surgical work, in which it is often desirable to determine the character of the organism present in a pus-formation or exudation without waiting to summon a bacteriologist or to collect the necessary cover-glasses or slides, culture-tubes,

FIG. 17.—Sterilized test-tube and wire swab for collecting pus and fluids for bacteriological examination (Warren).

[1] Wooden sticks for the swabs may be purchased under the name of "wood applicators."

platinum needle, etc. The swabs and their test-tubes may be kept on hand in a sterile condition, so that they may be handled by the operator or an assistant.

3. Fluid material may be collected by aspiration or otherwise. In the case of fluids care should be taken that everything with which the fluid comes in contact be clean and sterilized by heat if possible. The use of antiseptics, such as carbolic acid or corrosive sublimate, is to be avoided.

If a hypodermic syringe is used in obtaining material, it should be of a construction which will admit of sterilization by heat, and it should be so sterilized before using.

In the collection of pathological fluids, especially peritoneal exudates, a special form of apparatus has been found most useful. It consists essentially of a glass tube, about 14 cm. long and about 7 mm. in external diameter, one end of which is narrowed to a small opening and rounded off, while to the other end is attached a small rubber bulb like that on a "medicine dropper." It is to be kept ready for use in a test-tube, stoppered with cotton (Fig. 18), the whole having been sterilized as are surgical dressings. The rubber

Fig. 18.—Apparatus for the collection of pathological fluids.

bulbs are not expensive. Any number of pieces of this apparatus may be kept on hand in sterile condition.

When it is desired to obtain a sample of peritoneal or other fluid for bacteriological examination, the apparatus is removed from the test-tube and the fluid aspirated into it by manipulation of the rubber bulb. It is then replaced in the test-tube. The fluid thus obtained should be free from contamination and may be readily transported to the laboratory for examination.

Choice of Media.—The demonstration of the presence of bacteria in a tissue or exudate by means of culture consists in bringing a small amount of the material to be examined in contact with some nutrient substance on or in which the bacteria will thrive. The choice of culture-media depends upon the source of the material and the nature of the suspected organisms. Smear preparations from the material should be made at once and stained by Gram's method. Examination will give some idea as to the bacteria present. For routine work it is advisable to culture to blood agar plates, blood serum, and bouillon. These three media suffice for the cultivation and isolation of the majority of the pathogenic bacteria. If the original material contains several varieties of organisms it will be necessary to make subcultures from the original cultures in order to obtain pure cultures of the various kinds. Cultivation of certain organisms, such as the anaërobic bacilli, Gonococcus, B. tuberculosis, B. pertussis, and others, requires special media, directions for which will be found in the sections devoted to these organisms. In using blood agar plates a great saving can be made by dividing the under glass surface of the half containing the medium into quadrants with a wax pencil. By confining the surface smear of each specimen to the area of one of these quadrants four specimens can be cultured on a single plate. It is inadvisable to culture in this way material from chronic discharging ears or foul smelling pus from any source. Very frequently such material contains an organism belonging to the "proteus" group which rapidly spreads completely over the surface of the blood agar and contaminates the cultures in the other quadrants.

Method of Preparing Cultures.—The preparation of cultures consists in transferring to the medium in a test-tube as much of the tissue or other material as will adhere to the end of a piece of stiff platinum wire hammered flat at the end or a platinum loop. The former is fixed in the end of a glass or metal rod, and should be about 8 cm. long. It should have a rounded spatula-like extremity, and should be thick enough not to bend easily. The "platinum loop" consists of a piece

of platinum wire of about 22 gauge, $2\frac{1}{2}$ to 3 inches long, fixed
in the end of a small glass or metal rod 8 or 10 inches long.   The
tip should be bent around into a simple loop 2 to 3 mm. in
diameter.

In culturing, the tube containing the material and the culture-
tube that is to be infected from it are held side by side in the
left hand in a slanting position in such a way as to give a good
view to the operator of the surface of the media, while the
cotton stoppers are removed and held between the fingers of
the same hand (Fig. 19).   One
object of holding the tubes in a
slanting position is to offer less
chance of contamination from
bacteria gaining entrance to the
culture-medium from the air.

Some workers take further
precautions against contamina-
tion by either directing the
flame of the Bunsen burner on
the mouths of the tubes after
removing the stoppers or singe-
ing the cotton stoppers before
removing, or both.

FIG. 19.—Method of holding tubes
during inoculation.

The platinum wire, which is manipulated by the right hand,
is first sterilized by holding in the Bunsen flame until it glows
and then cooled by contact with the media to be inoculated,
after which its free end is carefully brought in contact with the
material to be inoculated, and immediately inserted into the
tube containing the culture medium.   The manner of inoculat-
ing the sterile culture-medium with the infected platinum wire
will vary with the form and character of the culture desired.

If the medium to be inoculated is a fluid one, the wire is
simply immersed in it and moved back and forth once or
twice.   If the medium be a solid one in the form of a slant,
the infected end of the wire is drawn over the surface once or
twice from the bottom of the slant to its upper end; or if the
solid medium in the tube be arranged for a stab culture (see

page 240), the infected wire is to be plunged once through the center of the mass to the bottom of the tube. After the tubes have been inoculated as above indicated, the wire is to be immediately withdrawn and sterilized in the flame and the cotton stoppers replaced in the tubes. They are then to be placed in the incubator for development. Gelatin cultures, however, must not be so treated, but are to be kept at room temperature, for the heat of the incubator would cause the gelatin to become fluid.

If the material is on a "swab," the medium may be conveniently inoculated directly by gently rubbing the swab over it. In this case it is usually best to make a dilution or two in sterile salt solution or bouillon if there be a large amount of material on the swab or if the smear examination has shown that a large number of bacteria are present. In any case it is important that the inoculated material be spread over all of the surface of solid media, and not in the form of one or two narrow streaks.

It is, of course, essential that the material brought in contact with the culture-medium should be free from bacteria not originally present in it. Therefore, in taking material from the interior of organs and tissues the surface is first sterilized by searing it with a hot knife, such as an ordinary case-knife, which has been heated in the Bunsen flame, and then, through a small incision made with another hot knife in this seared or sterilized area, the material from the interior is collected on the end of the platinum wire, which has also been previously heated in the Bunsen flame to sterilize it, and then cooled either by placing it in or on the media for a few seconds or by moving it about in the interior of the tissue.

In the case of exudations on free surfaces, however, this searing is impossible, and therefore care should be exercised at the autopsy not to contaminate any such exudate by handling before the material for culture has been obtained with the platinum wire. The material thus secured is then transferred by means of the platinum wire to the culture-tube. The infected wire should be gently rubbed over *all* of the surface of solid media, avoiding, however, the breaking of the surface. *It is*

important that the material be well distributed over the nutrient surface. If the material is suspected of containing a large number of bacteria, as in the case of suppurations or acute inflammatory lesions, it is advisable to make the first inoculation into a tube of bouillon. The routine cultures should be made from this bouillon dilution. This operation is called "diluting." The object of this is to obtain, after the development of the cultures, a sufficiently small number of colonies, so that they may be discrete—*i.e.*, separated from one another—and thus be enabled to exhibit their characteristic appearances, which are largely lost when the colonies are so numerous as to be confluent.

If thought desirable, a second bouillon dilution may be similarly inoculated from the first, but this is rarely necessary. As a rule, one tube will be sufficient to obtain discrete colonies from organs or tissues in which no suppurative or exudative condition is present.

Anaërobic cultures are indicated in certain cases. For anaërobic methods, see pp. 254 to 261.

After the manner above indicated cultures are to be made at autopsy as a matter of routine from the *blood of the heart,* the *liver,* the *spleen,* the *lung,* and the *kidney.* Cultures are also to be made from any acute inflammatory lesions in any situation.

As each culture-tube is inoculated it is to be labeled with the laboratory number, with the name of the organ or of the material from which it was inoculated, and with the date. For this purpose a wax pencil or small paper labels are used.

The culture from the blood of the heart should be made before the removal of that organ from the body, by searing the right ventricle and then puncturing it with a sterilized knife to admit the platinum wire. The amount of blood used for the culture should be as much as will adhere to the platinum wire. Cultures from vegetations in acute endocarditis are not usually of much value unless they are sufficiently large to enable a sterilization of their surface to be effected and material for culture secured from their interior.

Most pathogenic bacteria grow best at body temperature. Therefore, cultures in most instances are placed in an incubator at 37.5° C. and examined after twenty-four hours, or when the colonies have grown out upon them. The identification of the infecting bacteria present in most cases may be made from a consideration of the size, color, and general appearance of the colonies when taken in connection with the morphology of the bacteria composing them. In some instances, however, this may not be sufficient evidence upon which to base the diagnosis, and it may be necessary to obtain further facts in regard to a given organism in order to identify it with a sufficient degree of certainty. Thus it may be necessary to observe the appearances of its growth in pure culture in various media, and to ascertain whether it produces certain chemical changes in the media by its growth. Its ability to grow with or without oxygen, its reaction toward staining agents, whether it has independent motion or not, and its effects upon animals by inoculation, are also points which may have to be determined to enable one to make a positive diagnosis of the species to which the organism belongs.

Blood Cultures.—The blood should be withdrawn from an arm vein with a syringe under the strictest aseptic precautions, and should be immediately expelled into the medium. Ordinarily bouillon, contained in Erlenmeyer flasks, is the medium of choice. One part of blood should be added to each 20 parts of bouillon. If the presence of certain organisms is suspected it becomes necessary to use other media. For example, dextrose bouillon should be used for suspected streptococci, ascitic fluid should be added for suspected gonococci, and dextrose bouillon or agar followed by anaërobic incubation for suspected anaërobic bacilli. Frequently an idea as to the actual number of organisms per cubic centimeter of blood is important to the clinician. This is easily determined by adding 1.0 c.c. of blood to a tube of melted agar cooled to 40°–45° C. The contents are immediately mixed and poured into a sterile Petri dish. If possible all cultures should be made at the bedside. For flaming the various media containers

an alcohol lamp can be used. In case it is impossible to make the cultures at the bedside the blood should be expelled into a test-tube containing a 5 per cent. sterile solution of sodium citrate, in the proportion of 9 parts of blood to 1 part of citrate solution.

Stock Cultures. — The biweekly or weekly subculturing of stock cultures of the less fastidious bacteria and the more frequent transplants required for the maintenance of such organisms as meningococci and gonococci demand a great deal of time, provided numerous strains are being carried along. Furthermore, by frequent subcultures without animal passage the virulence of such organisms as pneumococci and strepto-cocci is greatly decreased, and frequently certain biological characteristics are lost. It is well known that bacteria may be dried after freezing without causing death, and Swift[1] has utilized this fact in devising a simple method for the preservation of stock cultures. A heavy suspension of a young, active culture is pipetted in small amounts into several test-tubes (110 × 5-10 mm.). These are placed in a desiccator which is surrounded by a salt and ice mixture. The lower part of the desiccator contains glycerine in which the tubes are immersed. As soon as the suspension is well frozen the lid is placed on the desiccator and the air exhausted, first with a water pump and later with a high vacuum pump. When a high vacuum (2-3 mm. of mercury) has been obtained the stop-cock is closed, and the desiccator is disconnected, removed from the salt-ice mixture, and placed in an ice box until desiccation is complete (about twelve hours). The tubes are removed, plugs pushed down, and sealed *completely* with paraffin. They can be stored at room temperature, preferably in the dark.

Using this method streptococci were recovered up to forty months after drying without loss of virulence or change in sugar fermentations. Living meningococci were recovered two months after desiccation of 0.2 c.c. of broth containing one loopful of bacterial growth.

Methods of Obtaining Pure Cultures. — If there is a more or less confluent growth of colonies of various kinds in a culture-

[1] Swift, *Jour. of Exp. Med.*, 1921, xxxiii, 69.

tube, and it is desired to isolate a pure culture of one of the
species of bacteria present, it is obvious that the first step is to
obtain separate or "discrete" colonies of that organism.   This
may be accomplished by any of the so-called "dilution"
methods.   One of these methods has already been mentioned
in connection with the making of cultures from material
containing many organisms (see page 249).

Another very satisfactory method is that of "plating."   For
this purpose *Petri dishes* are used.   These consist of clear glass
circular dishes 10 cm. in diameter and about 1 cm. deep.   A
loose fitting glass cover of about the same depth and slightly
greater diameter fits over the top of each.   The two halves are
placed together, wrapped singly or in lots of three or four in
paper, and sterilized in the hot air sterilizer.   Three tubes of
agar are melted by immersion in boiling water and cooled by
placing in a water bath at 40°–45° C.   If gelatin is used a tem-
perature of 35°–40° C. is sufficient to cause liquefaction.   The
tubes are then inoculated successively from the bacterial growth
or from the pathological material from which it is desired to
obtain discrete colonies.   The first tube is inoculated from the
growth or tissue, the second from the first tube, by transferring
with the platinum loop one or two loopfuls, and the third from
the second tube by transferring three or four loopfuls.   After
inoculating each tube the contents are thoroughly mixed by
vigorously rolling the tube held in an upright position between
the palms of the hands.   After taking the agar tubes out of the
water bath too much time must not be taken; for, if the tubes
cool below 38° C., the agar will solidify.   The contents of the
tubes are then poured into the Petri dishes by removing the
cotton stoppers, carefully flaming the lips of the tubes, and lift-
ing the Petri dish covers on one side a sufficient height to admit
the mouths of the tubes.   The lips of the tubes should not be
allowed to touch either the bottoms or covers of the Petri dishes.
The dishes containing agar, or agar-plates as they are usually
called, are allowed to harden at room temperature and are then
placed upside down in the incubator at 37.5° C.   This inversion
is to prevent the vapor of the water squeezed out of the agar

during hardening from condensing on the under side of the cover and dropping down on the surface of the agar. The same object is accomplished without inverting the dishes by using covers made of porous earthenware or by placing in the cover before sterilization a circular piece of filter paper of slightly greater diameter than that of the cover. Growth on the plates is much more readily observed if clear glass covers are used. Gelatin plates are allowed to harden, and are put away in the dark at room temperature or in a special incubator at $20°-25°$ C.
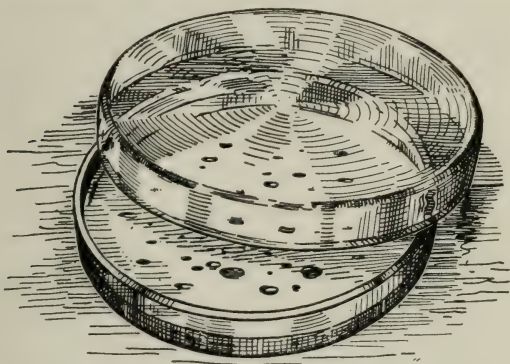
FIG. 20.—Petri dish with colonies.

Colonies usually develop in the agar-plates in twenty-four hours. Gelatin plates require somewhat longer incubation. In the second or third plate it will usually be possible to "fish" individual colonies. This is to be done by touching the desired colony with the tip of a sterile platinum needle, and transferring to suitable media. This "fishing" is most accurately controlled by placing the lower part of the dish on the stage of a microscope and observing the actual process through a low power objective (Zeiss AA or Leitz No. 2).

Frequently it is necessary to isolate an organism which grows best on certain special media, such as blood or ascitic agar. In such instances the blood or ascitic fluid may be added to the agar after melting and before inoculating. However, it is usually more convenient to use the "streak plate" method. A suitable medium is prepared and poured into Petri dishes.

After hardening, a loopful of the material to be cultured is transferred to an area the size of the loop at the margin of the surface of the medium. The platinum loop is burned off and allowed to cool. The material is then spread over the surface of the plate with the platinum loop either by radial streaks starting each time from the original point of inoculation or by a continuous zig-zag streak covering the whole plate surface starting from the original point and continuing to the opposite side. Without burning off the loop again it is then streaked over the surface of a second plate, or even a third. A modification consists in making primary dilutions in bouillon or sterile salt solution and streaking the various dilutions each on a single plate.

Cultivation without Oxygen (Anaërobic Cultures).— A condition of anaërobiosis may be established by various means, such as, by the mechanical exclusion of oxygen from oxygen-free media, by the chemical absorption of oxygen, by the replacement of air by inert gases in an air-tight container, by the combination of the latter method with combustion of the contained oxygen, by the presence of reducing substances in the media, or finally by combinations of these methods.

Before taking up in more detail these various methods some of the fundamental facts should be considered. Gates and Olitsky[1] have recently reinvestigated the factors influencing anaërobiosis produced by a combination of two of these methods —namely, the exclusion of oxygen by mechanical means from oxygen-free media and the presence of reducing substances (dextrose, peptone, and animal tissue). Their work showed that liquid paraffin oil overlaid on the surface of oxygen-free fluid media does not prevent the access of oxygen, whereas solid vaseline forms an effectual seal. The length of the column of medium is not important under a vaseline seal. The presence of animal tissue (0.6 gm. rabbit kidney per tube), dextrose (2 per cent.) or, to a lesser degree, peptone each in faintly alkaline (pH 7.2–7.4) bouillon, serves to maintain, and in the first instance locally to establish, a condition of anaërobiosis because

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[1] Gates and Olitsky, *Jour. of Exp. Med.*, 1921, xxxiii, 51.

of their content of reducing substances. Infusion broth, ascitic fluid, and animal serum alone do not contain appreciable amounts of such substances. Semi-solid (peptone broth containing 0.25 per cent. agar) oxygen-free media without sealing so decreases the penetration rate of atmospheric oxygen that the slight reducing action of the peptone is able to maintain a condition of anaërobiosis in the lower four-fifths of the tube. Their findings in regard to the reducing powers of dextrose and peptone corroborate the recommendations of many workers to use for anaërobic cultures bouillon (peptone broth), gelatin, or agar each containing dextrose and with a slightly alkaline reaction (slightly acid to phenolphthalein). So far as anaërobiosis is concerned their work would indicate that it is not necessary to add animal tissue; however, such tissue may, in fact very probably does, add to the medium substances which favor the growth of certain anaërobes.

It is best to immerse all tubed media used for anaërobic cultures in boiling water for ten or fifteen minutes in order to expel absorbed oxygen. The tubes should be cooled rapidly (agar to about 45° C.) and inoculated within a few minutes.

As an indicator of anaërobiosis enough of a 1.0 per cent. aqueous solution of methylene blue should be added to 2 per cent. dextrose peptone broth (pH 7.4–7.8) to give a distinct blue color (about 2 drops for each 10 c.c. of broth). In the absence of oxygen the dye is gradually reduced to colorless leucomethylene blue. The reaction is reversible, and in the presence of oxygen this colorless derivative is very rapidly oxidized to the original compound. Such tubes should be placed in the jars along with the culture tubes and plates. If jars are not used, the methylene blue should be added to the medium used and the mixture treated in the same way as the culture tubes.

Mechanical Methods.—*Liborius'* method is an example of the exclusion of oxygen by mechanical means. This consists in cultivating the bacteria in the depths of oxygen-free (previously boiled) solid media in test-tubes filled to a considerable height, so that oxygen cannot penetrate to them through the

thick layer of medium (Fig. 21). The weak reducing action of dextrose and peptone in the medium counteracts the invasion of oxygen at least in the lower half of the tube. The additional precaution of a vaseline seal permits the use of fluid media and furnishes by far the simplest of the anaërobic culture methods and one which is highly recommended. The vaseline should be melted in a rather high-walled metal or agate-ware cup and heated until it commences to fume and boil ($180°$–$190°$ C.). This temperature is sufficient to kill any bacteria present, including spore bearers. The vaseline is allowed to cool

FIG. 21.—Liborius's method of making anaërobic cultures.

FIG. 22.—Buchner's method of making anaërobic cultures.

(below $100°$ C.) and is then transferred to the culture-tube to a depth of about 1.0 cm. by means of a sterile pipette. The vaseline should be heated a sufficient time ahead to permit adding it to the media *immediately* following inoculation.

In using a solid medium the colonies may be made accessible for subcultures either by breaking the tube or by removing the overlying portions of the culture-medium by means of a stout platinum wire, previously sterilized. The microscopical appearances of the colonies may be studied by placing thin slices of the medium, containing the colonies, on a slide. If dilutions

(see page 249) are made, this method will be found very practical for obtaining pure cultures from mixed growths. In making stab cultures the agar or gelatin after heating is rapidly cooled and solidified by immersion in ice water.

Chemical Absorption Methods.—*Buchner's method* consists in cultivating bacteria in an atmosphere from which the oxygen has been absorbed by a mixture of alkali and pyrogallic acid. Tube-cultures, or cultures in Petri dishes, may be used. They should be placed in some form of a glass chamber, which is closed air-tight, along with the necessary quantity of alkali and pyrogallic acid mixture. In preparing the apparatus, the pyrogallic acid (in powder) is placed first in the chamber along with the culture tubes or plates, then the necessary quantity of a solution of potassium hydroxid (1:10) is run in, and the chamber quickly closed. For single tube-cultures a large test-tube provided with a tightly fitting rubber stopper, which is sealed in position with wax, may be used for the air-tight chamber (see Fig. 22). The culture-tube is to be elevated above the surface of the reducing mixture by means of a bent wire.

It is necessary to seal up the apparatus quickly in order to obtain the full benefit of the oxygen-absorbing power of the pyrogallic acid. The quantity of pyrogallic acid employed should be about 1 gram for each 100 c.c. of air-space to be exhausted of oxygen, and for every gram of pyrogallic acid 10 c.c. of the solution of potassium hydroxid should be used.

Wright's method depends upon the absorption of oxygen by an alkaline solution of pyrogallic acid, as in the well-known method of Buchner. It is applicable to culture in test-tubes and in flasks. The details of the method are as follows:

After the culture-medium in the test-tube has been inoculated, the cotton stopper is thrust sufficiently far down into the test-tube so that the upper end of the cotton stopper lies about 15 mm. below the mouth of the test-tube. It is usually desirable to cut off a part of the protruding portion of the cotton before doing this. Now fill the space in the

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tube above the cotton stopper with dry pyrogallic acid.   Next
pour quickly onto this pyrogallic acid enough of a strong watery
solution of sodium hydroxid to dissolve it all; avoid pouring
on an excess; for a test-tube $\frac{3}{4}$ inch in diameter about 2
c.c. will be an ample quantity.   Then, as quickly as possible,
insert firmly a rubber stopper in the
mouth of the tube so as to close it
tightly.   The culture is then ready to be
set aside for development.

The cotton of the stopper should be
of a kind that will readily absorb fluids.

The solution of sodium hydroxid con-
sists of one part of sodium hydroxid in
sticks and two parts of water.

It may be thought that there is danger
of contaminating the culture-medium
from the alkaline pyrogallic acid mixture
running down the sides of the tube.
This does not occur because the mass of
the cotton stopper is sufficiently large to
absorb completely the quantity of fluid in
it, with a good margin to spare.

This simple method has given satis-
factory cultures of the tetanus bacillus
obtained from cases of tetanus in the
Massachusetts General Hospital and of
other obligate anaërobic bacteria.   It can

Fig. 23. — Wright's
method for the cultivation
of anaërobes.

be applied to all forms of test-tube cul-
tures, both in solid and fluid media,
including Esmarch roll-cultures.   In ap-
plying the method to Esmarch roll-cultures the mixture of
pyrogallic acid and alkali should be placed in the cotton, and
the rubber stopper inserted before the tube is rolled on the ice.
Glucose-agar readily lends itself to Esmarch roll-cultures if the
tubes are kept in a slanting position during growth.

The accompanying photograph shows the appearance of a
boullion tube prepared according to this method (Fig. 23).

Oxygen Displacement Methods.—Inoculated tubes or Petri dishes are placed in a suitable air-tight chamber and the contained air is displaced either by passing through it a stream of hydrogen or nitrogen for ten or fifteen minutes or by exhausting the air, and admitting hydrogen or nitrogen, repeating the process two or three times. Both hydrogen and nitrogen can be obtained in tanks from commercial houses. Before admitting either gas to the culture chamber it should be passed through several wash bottles containing alkaline pyrogallol to remove any traces of oxygen.

Combined Oxygen Displacement and Combustion Methods.—In general this method is dependent upon the fact that freshly heated finely divided platinum or palladium acts as a catalyzer for the union or combustion of hydrogen and oxygen to form water. McIntosh and Fildes[1] were the first to utilize the process for the cultivation of anaërobes in jars. They suspend from the jar-cover a bit of asbestos impregnated with platinum or palladium black and wrapped in a piece of wire gauze. After filling the jar with the tubes or plates to be incubated, the basket containing the asbestos is heated in the flame of a Bunsen burner, and the cover is quickly placed on the jar. Hydrogen is then admitted slowly through an opening in the cover. The oxygen under the influence of the catalyzer unites with the hydrogen to form water, and this process continues until all of the oxygen is used up. The hydrogen must be introduced into the jar before the asbestos cools, otherwise combustion does not take place.

Brown[2] has recently modified their technic by substituting for the wire basket containing the asbestos an arrangement whereby the asbestos is heated by a coil of wire carrying an electric current. This unit is surrounded by a cylinder of fine copper wire gauze, which serves, as in a Davy safety lamp, to minimize the danger of explosion. Heating the asbestos in this way does not require speed in assembling the jar and in addition permits the removal of traces of oxygen any time subsequent to

[1] McIntosh and Fildes, *Lancet*, 1916, i, 768.
[2] Brown, *Jour. of Exp. Med.*, 1921, xxxiii, 677; and 1922, xxxv, 467.

the primary combustion. In our hands this method has proved most satisfactory and is recommended for anaërobic plate cultures. The details are as follows:

An ordinary round heavy glass museum jar of any desired size is used. It should be provided with a metal clamp for holding down the cover, and a hole about 2.0 cm. in diameter should be bored in the center of the cover. Take a piece of soft glass tubing (4–5 mm. outside diameter) about 10 cm. long and blow out a small hole about 2.5 cm. from each end. Take a piece of fine nichrome wire (B. & S. #28) about 35 cm. long and fasten to each end by means of a drop of solder a piece of bare copper wire (B. & S. #22) about 30 cm. long. Cut out a piece of lens paper 4 cm. wide and as long as the distance between the two holes in the glass tube (about 5 cm.). Spread out the asbestos[1] evenly over the paper and wrap it firmly about the glass tube between the two holes. Hold the paper in place by coiling around it the nichrome wire, leading out the two copper wires through the two holes and the lumen to the ends of the tube. Place at each end of the tube a one-hole #5 rubber stopper, allowing the tube to protrude 5 mm. at each end. The space between the two proximal ends of the stoppers is closed in by wrapping about the stoppers a cylinder of very fine copper or brass wire gauze well overlapped and held by wires about the stoppers. The twisted ends of these wires are carried up to the two glass hooks on the under surface of the jar-cover and are fastened so that the unit is held 3–4 cm. below the cover. The lumen of the glass tube at each end where the copper wire emerges should be packed tight with plain asbestos. Place on each copper wire a piece of fine rubber tubing of sufficient length to cover the wire from the end of the glass tube to the hole in the jar-cover, wrapping the joints at the ends of the glass tube with insulating tape. Fit a one-hole rubber stopper to the hole in the jar-cover and pass the copper wires through the stopper, one on each side of the hole, by passing through a large hollow bore needle from the upper side, threading the wire through the lumen of the needle, and withdrawing the needle. Pull the copper wires through so that inside the jar they are completely covered by the rubber tubing. Insert a glass stop-cock into the hole of the rubber stopper and to the lower end attach a piece of rubber tubing of suffi-

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[1] Asbestos impregnated with platinum or palladium black may be obtained from any of the larger commercial firms. Palladinized asbestos is preferable. It may easily be prepared by allowing purified long fibered asbestos wool to absorb the maximum amount of a 5 per cent. solution of palladium chloride, adding a solution of ammonium chloride in excess to form the double salt, evaporating to dryness over a water bath, and igniting in a small porcelain crucible over a blast lamp.

cient length to reach to the bottom of the jars. To prevent leaking between the stopper and the cover seal generously with sealing wax or Major's cement. By fitting a piece of thin sheet asbestos to the underside of the glass cover the possibility of the heat of combustion cracking the cover is greatly diminished. This is held in place by dabs of sealing wax.

For use, the jar is filled with the tubes or plates to be incubated and then both the top of the jar and the lip of the cover are covered with a thin even layer of modelling clay ("plasteline"). The cover is placed on the jar, very firmly pressed into position, and the clamp applied. The two copper wires are connected to a 110 volt electric light circuit with *one* 60 watt lamp in series. Hydrogen is run into the jar under about 5 lbs. pressure. Combustion is indicated by the formation of a fine mist inside the jar and the deposition of moisture on the inner walls. After twenty or thirty minutes the cessation of the flow of hydrogen into the jar, as evidenced by lack of bubbling in the wash bottles, indicates that combustion is complete. Close the stop-cock, disconnect the wires, and incubate. Each jar should contain a control tube of medium containing methylene blue, decolorized by boiling prior to placing in the jar.

Animal Tissue Methods.—Fairly deep tubes of dextrose bouillon, agar or gelatin containing pieces of fresh sterile animal tissue (rabbit or guinea-pig kidney, spleen or liver) are often used for anaërobic cultures. Such tissues contain certain reducing substances which diffuse out into the surrounding medium and serve to maintain an oxygen-free zone. The height of the zone produced by a constant amount of tissue depends upon the rate of oxygen penetration from the surface of the medium. It is also probable that nutrient substances also diffuse out into the surrounding medium, for numerous investigators, most notably Noguchi, have found this to be the method of choice for the anaërobic cultivation of certain organisms.

The Inoculation of Animals.—The animals ordinarily used in the laboratory are guinea-pigs, rabbits and mice. The instruments, etc., used in the inoculation of animals should be sterilized beforehand, but strict surgical asepsis is not necessary as a rule.

The quantity of bacteria used for purposes of inoculation varies with the organism and with the end in view. In general, it may be said that in inoculating with the growth from

a solid medium with the platinum wire one or two loopfuls are used. If bouillon cultures are employed, the quantity injected varies from 0.1 c.c. to 1 c.c. in most cases.

In cases where a "suspension" of the growth on a solid medium is injected the same quantities are used as in the case of bouillon cultures, the density of the suspension depending upon the operator. A "suspension" may be conveniently prepared by pouring 5 or 8 c.c. of sterile bouillon, sterilized water, or 0.6 per cent. sodium chlorid solution (sterilized) into the tube containing the growth upon solid medium, then breaking up the colonies of the growth with the platinum wire, and shaking the tube.

Guinea-pigs are in most instances inoculated either subcutaneously or into the peritoneumal cavity.

Subcutaneous inoculation is effected either by injection with a hypodermic syringe or by the introduction of the material to be inoculated through a small incision in the skin. The best point for subcutaneous inoculation is the tissue of the anterior abdominal wall. In inoculating large amounts of fluid (4 to 5 c.c.) it is advisable to insert the needle into the abdominal wall muscles 1 or 2 cm. to one side of the median line, continuing across the median line and making the injection in the subcutaneous tissue 2 or 3 cm. across on the opposite side. By employing this method leaks very rarely occur.

In inoculating, the animal is to be held abdomen uppermost on an animal board or by an assistant, who grasps the neck and fore quarters with one hand and the hind quarters with the other. If the skin is to incised, the hair about the point of inoculation is to be cut short with a pair of scissors and the skin cleansed with soap and water. An incision is then to be made about 8 or 10 mm. long through the skin, including the subcutaneous tissue, and the superficial tissues separated from the muscle for a distance of 10 or 15 mm. toward one side of the wound by inserting the points of scissors or other instrument, so as to form a "pocket" beneath the skin. In this "pocket" the material for inoculation is introduced, either on the platinum wire or by means of small forceps.

If pieces of tissue are used, it may be well in some cases to close the wound by one or two sutures in order to prevent the extrusion of the material after the release of the animal.

Intraperitoneal inoculation may be performed essentially as above indicated. If the inoculation be by incision, the opening into the peritoneal cavity should be as small as possible, and the wound should be firmly closed with silk sutures in order to prevent extrusion of the intestines. In inoculating with the hypodermic syringe the needle should not be pushed in too far or the intestines may be wounded. The needle is best introduced a little to one side of, or slightly below, the umbilicus.

In the hands of the uninitiated *intracardiac inoculation* is a difficult procedure and one fraught with great danger for the guinea-pig. The needle is inserted between the ribs on the left side over the area of maximum cardiac impulse and is pushed in very slowly until the appearance of blood in the barrel of the syringe indicates that one of the heart chambers has been tapped. The animal should be held very firmly on its back by an assistant. After a few attempts, and probably nearly as many failures, the technic is mastered. This method offers a practical way of obtaining an appreciable quantity of blood from a guinea-pig without killing the animal. From 5 to 8 c.c. of blood can be taken from an adult guinea-pig without causing any ill effects.

Rabbits.—These animals may be inoculated both subcutaneously and intraperitoneally, essentially as described for guinea-pigs.

During the operation of inoculating, the assistant grasps the ears with one hand and the hind legs with the other, while the body of the animal rests upon the table, abdomen uppermost. Rabbits held for a few seconds in this position usually become perfectly quiet, and often do not show any evidence of pain during the operation.

Intravenous inoculation is usually done on rabbits, because of the ease with which the needle of a hypodermic syringe may be introduced into the long and prominent marginal

vein of the ear. In inoculating in this manner the tip of the ear is held by the thumb and fingers of the left hand, while the right manipulates the syringe, the needle of which is pushed through the skin of the external surface of the ear into the vein which runs along the outer margin of the ear (Fig. 24).

By the exercise of care and gentleness the animal may be thus inoculated without being held by an assistant, especially if the fur between the ears be stroked for a short time just
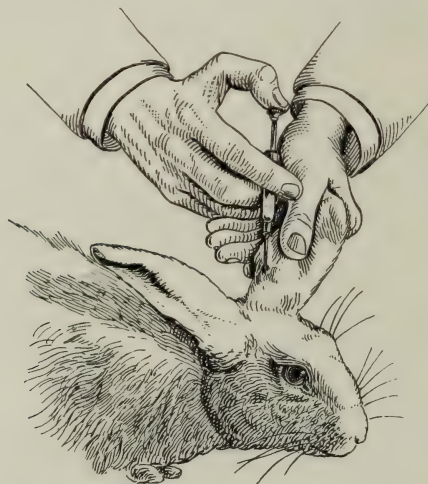
FIG. 24.—Method of making an intravenous injection into a rabbit. Observe that the needle enters the posterior vein from the hairy surface (McFarland).

before the introduction of the needle. In some cases it may be necessary to anesthetize the animal on account of violent struggling. (See below.)

Intracardiac inoculation is but rarely done on rabbits because of the ease with which injections can be made into the blood stream via the ear veins. However, cardiac puncture furnishes an excellent means of obtaining moderately large amounts of blood. The actual technic is the same as with guinea-pigs. From an adult rabbit up to 40 to 50 c.c. can be withdrawn.

Injection of bacteria into the mesenteric veins by means of the hypodermic syringe, after laparotomy, may be performed both on rabbits and on

guinea-pigs. This is to be done under anesthesia. Ether is very satis-factory for this purpose. Guinea-pigs bear it well, but it is to be used with caution on rabbits. With the latter animals death is liable to occur if the ether is "pushed" after complete anesthesia is established. Rabbits once thoroughly anesthetized seem to remain so for a considerable time without additional ether being necessary. The incision for this form of inoculation should be in the lower half of the abdominal wall in the median line, for in this region the coils of the small intestine are most numerous. The length of the incision should be about 2 cm. Several loops of intestine are brought out through the wound, and a mesenteric vein, of the proper size to admit the needle of a hypodermic syringe, is sought for. When found the needle is to be introduced and held firmly in position while an assistant carefully presses inward the piston of the syringe. After the injection of the material the needle is withdrawn, the punctured vein picked up with the artery-forceps, and the vessel tied on both sides of the puncture with silk thread. The loops of the intestine are then replaced and the wound closed in two layers, one consisting of the muscles and peritoneum, the other of the skin. The so-called "button-hole stitch" with silk thread is very well fitted for the closing of the wound.

Little or no aseptic precautions are necessary to obtain primary union in the wound. Before the operation, however, the hair of the region should be cut off close and the skin cleansed with soap and water.

This form of inoculation may be useful in studying the local effects of bacteria upon liver-tissue, for large numbers of them will be lodged in the capillaries of the liver, and mi-croscopical sections of any part of the organ will contain them, so that any local lesion pro-duced by them may be sub-jected to observation after variable intervals of time.

Mice may be inoculated subcutaneously or intra-peritoneally.

Subcutaneous inoculation is usually made at the root of the tail. The animal,

FIG. 25.—Mouse-holder, with mouse in position for subcutaneous inoculation.

manipulated by means of chemists' crucible tongs or a similar instrument grasping his tail, is to be persuaded to crawl into a cylinder of wire gauze, about 8 to 10 cm. long and about 3 cm. in diameter, which is fixed on a small board. The

cylinder is open at both ends, and when the mouse has crawled into it—a thing which he will readily do—the end near his tail is bent inward so as to prevent him from backing out of it, while an ordinary small screw-clamp is adjusted firmly to his tail to prevent his escaping through the other end. The animal is thus secured and ready for the operation of inoculation. A more complete form of this apparatus, with a fixed clamp for the animal's tail, is shown in Fig. 25.

In making the inoculation the mouse is pulled backward by the tail until his rump is exposed in the end of the cylinder, and then with small scissors the hair is cut away over a space, approximately 1 cm. square, about the root of the tail. In the center of this a small opening is made through the skin 3 or 4 mm. long with small scissors, and through the opening the points of the scissors are passed anteriorly beneath the skin for a distance of about 1 cm., so as to make a "pocket" or cavity by separating the skin from the muscles. Into the cavity thus formed the material for inoculation is then to be introduced by means of the platinum wire. As a rule, white mice are to be preferred to the wild brown variety, on account of the greater ease with which they may be handled.

Intraperitoneal inoculation is most conveniently performed while holding the mouse in the left hand. The mouse is removed by its tail from its jar or cage by means of tongs or long forceps and is placed preferably on a cloth surface. The tail is grasped with the right hand and the skin at the nape of the neck is caught *firmly* between the thumb and forefinger of the left hand. The mouse is lifted up, and the left hand turned so that the palm of the hand faces toward the manipulator. The tail of the mouse is held against the lower part of the palm of the left hand by the little finger. The back of the mouse rests on the second and third fingers, and by lowering or raising these two fingers the abdominal wall may be relaxed or drawn tight. The syringe is grasped in the right hand and the needle is inserted underneath the skin in the groin. The needle is passed upward a few millimeters sub-

cutaneously; then, the end of the syringe is tilted upwards and the needle is thrust into the peritoneal cavity.

The Care of Animals.—Inoculated guinea-pigs should be kept in boxes or cages so arranged as to permit of cleaning and disinfection. Cages made of a combination of galvanized-iron wire netting and galvanized sheet iron are to be preferred. The bottom of the cage should contain sawdust, and the top may be made to open on hinges. Good dimensions for such cages are 16 inches long, 10 inches wide, and 10 inches high. They may be satisfactorily disinfected, in most instances at least, by washing with boiling water.

Inoculated mice are well kept in large glass jars with perforated covers. A small amount of tissue paper should be provided for bedding.

The "stock" guinea-pigs and rabbits may be kept together in a pen which should have light and ventilation. Guinea-pigs breed readily and their young thrive, but this is not usually the case with rabbits. Mice may be kept for use in a woven-wire cage set in a sheet-iron pan, which will permit of the easy removal of excreta. Some raw cotton should be furnished for bedding. The young of white mice are difficult to raise to maturity.

Food.—Rabbits and guinea-pigs eat the same things. In summer-time, grass, green corn-husks, and green vegetables generally are good food for them. In winter, carrots and oats form a satisfactory diet. Fresh water should also be supplied.

Mice may be fed on stale bread soaked in water, oats, birdseed, and occasionally some cheese. Fresh water should be furnished, and, if possible, a little milk sometimes.

MICROSCOPICAL EXAMINATION OF BACTERIA

THE EXAMINATION OF THE LIVING CELL

Hanging Drop Preparations.—Chiefly for the purpose of determining the motility of bacteria the individual organisms may be observed, unstained, in a drop of bouillon or similar fluid under the oil-immersion lens. For this purpose a so-called *"hanging drop"* is prepared, for which a special form of slide known as a *"hollow slide"* is necessary. The hollow slide is a slide having a shallow circular concavity, about 1 cm. in diameter, ground out in its center (Fig. 26).
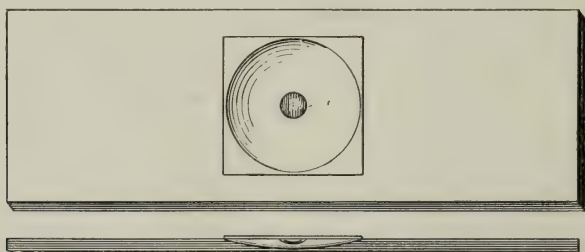
FIG. 26.—The "hanging drop" seen from above and in profile.

In preparing a hanging drop the procedure is as follows: A small drop of a bouillon culture or of the water of condensation of a blood-serum or agar slant is placed in the center of a cover-glass by means of the platinum loop. The cover-glass is then placed, drop downward, over the circular depression in the hollow slide. To hold the cover-glass in its place and to prevent evaporation of the fluid in which the organisms are suspended, a little vaselin is painted around the margin of the depression before placing the cover-glass in position. The hanging drop thus prepared is then examined by focusing

upon it with the oil-immersion lens, a small aperture of the iris diaphragm of the condenser being used to render the bacteria visible by refraction.   To facilitate focusing, the edge of the drop should be brought into the center of the field of the low-power objective, and then the oil-immersion put in place and focused upon it, the edge of the drop being more readily seen as a sharp line, owing to refraction, than the organisms.   Great care is necessary to avoid breaking the cover-glass in the effort to bring the bacteria into view.   Hanging drops may also be prepared from suspensions of bacteria grown on solid media, by mixing a portion of the growth with a small quantity of bouillon.

In the study of spore-formation the hanging drop is of great utility. Here the slide and cover-glass must be carefully sterilized before using, the cavity between the cover-glass and the slide well sealed with vaselin, and other precautions taken to prevent contamination of the drop with other bacteria.   The preparations may be placed in the incubator or on a "warm stage" and the process of spore-formation followed.

H. W. Hill's "Hanging-block" Method for the Observation of Developing Bacteria.—"Pour melted nutrient agar into a Petri dish to the depth of about one-eighth to one-quarter inch.   Cool this agar and cut from it a block about one-quarter inch to one-third inch square, and of the thickness of the agar layer in the dish.   This block has a smooth upper and under surface.   Place it, under surface down, on a slide, and protect it from dust.   Prepare an emulsion in sterile water of the organism to be examined if it has been grown on a solid medium or use a broth culture; spread the emulsion or broth upon the upper surface of the block as if making an ordinary cover-slip preparation.   Place the slide and block in a 37° C. incubator for five or ten minutes to dry slightly.   Then lay a clean sterile cover-slip on the inoculated surface of the block in close contact with it, usually avoiding air-bubbles.   Remove the slide from the lower surface of the block, and invert the cover-slip so that the agar block is uppermost.   With a platinum loop run a drop or two of melted agar along each side of the agar block, to fill the angles between the sides of the block and the cover-slip.   This seal hardens at once, preventing slipping of the block.   Place the preparation in the incubator again for five or ten minutes to dry the agar seal.   Invert this preparation over a moist chamber and seal the cover-slip in place with white wax or paraffin. Vaselin softens too readily at 37° C., allowing shifting of the cover-slip. The preparation may then be examined at leisure.   For Bacillus diph-

theriæ and organisms of similar size, Zeiss ocular 5, objective $\frac{1}{12}$, oil immersion, and a Welsbach light prove satisfactory, although a lower ocular and higher objective are better. The Abbé condenser is not used. If preferred, the Welsbach light may be concentrated by a four-inch lens, focal length seven inches. An incandescent electric lamp is very difficult to focus and does not yield good results." . . .

"Bacteria multiplying readily at room-temperature can be observed in such a preparation exactly as an ordinary hanging drop is observed, except that the slide should be secured rigidly in some way to the microscopic stage to prevent shifting. For bacteria growing best at 37° C. a warm stage is required."

THE STAINING OF BACTERIA IN SMEAR PREPARA= TIONS

Clean slides and cover-glasses are essential for the proper preparation of stained specimens. Only the best quality of slides and cover-glasses should be used. They may be cleaned by treating consecutively with soap-suds, cleaning solution (sulphuric acid and potassium bichromate) or 20 per cent. sulphuric acid, water, and alcohol. Very often simple immersion in alcohol will suffice. They are wiped dry with a soft cotton cloth or old linen handkerchief. It is recommended that cover-glasses be stored dry and ready for use protected from the dust by proper containers. Slides may be kept immersed in alcohol and wiped dry before use.

In making smear preparations from fluid media or body fluids the amount of fluid held by a small sterile platinum loop is spread out as thinly as possible on the slide or cover-glass. With frank pus it is usually advisable to dilute by previously placing a drop of sterile or freshly distilled water on the slide or cover-glass. In smearing from solid media a bit of the colony to be examined is picked up with a sterile platinum wire, thoroughly mixed with a small drop of water on the slide or cover-glass, and the suspension spread out as thinly as possible. Infected swabs and bits of tissue may be smeared directly with or without dilution, taking care that all desired cultures are made before smearing. As a rule the smear preparations of a

beginner contain so many organisms that the examination of individual bacteria is next to impossible. In preparing smears from material consisting chiefly or entirely of uncoagulated blood serum a thick layer of coagulated protein forms on fixing which frequently is washed off during the staining process. This may be prevented by diluting and by spreading the dilution over a large area.

The smears are allowed to dry in the air, and are then fixed, usually by heat. Cover-glasses are placed in cover-glass forceps smear side uppermost and are quickly passed through the flame of a Bunsen burner or alcohol lamp three times. Holding a slide with one's fingers first at one end and then at the other and passing it through the flame rather slowly three or four times in each position usually suffices for fixation. More accurate fixation with the disadvantage of the time required is obtained by immersion in one of the common fixing fluids—methyl alcohol, formalin, Zenker's fluid, etc.

In staining cover-glass preparations all of the various steps are made while holding the cover-glass with forceps. Slides may be held in the fingers or by forceps or may be placed in suitable staining dishes. Where a small sink is handy a very satisfactory arrangement consists in placing across the top from one side to the other two heavy glass rods fastened firmly 2–3 cm. apart at each end. To stain, the slides are laid on the rods. Wash water is obtained directly from the cold water faucet to which is attached a piece of flexible rubber tubing of suitable length.

After staining, the preparations are washed thoroughly with water, blotted with several layers of relatively smooth soft filter paper, and dried. The latter process may be hastened by passing through the flame.

For temporary preparations cover-glasses are mounted in water. This is done by placing the cover-glass film side down, with as much water as will adhere to it, on a clean slide and then removing all excess of water by gentle pressure with several thicknesses of filter paper. Permanent preparations are mounted in xylol colophonium or balsam, taking care that the

cover-glass is thoroughly dry before mounting. Slides are examined microscopically by placing a drop of cedar oil directly on the stained smear. For permanent preparations a drop of xylol colophonium or balsam is placed on the stained smear and a clean cover-glass dropped in place. If the slide has previously been examined without a cover-glass the cedar oil should be removed with xylol.

Simple Stains.—Used for the demonstration of bacteria in general, and also useful in gaining an idea of the character of the cellular elements in the preparation.

The simplest stains consist of 5 per cent. aqueous solutions of saturated alcoholic solutions of aniline dyes, such as, basic fuchsin, methylene-blue, methyl violet, and safranin.[1] Staining requires one-half to several minutes depending upon the affinity of the dye used. For simple staining several stains containing the above-mentioned dyes are most generally used. The most important are carbol-fuchsin, Löffler's alkaline methylene-blue and aniline methyl violet (see pages 77, 75, and 77). Löffler's alkaline methylene-blue is perhaps the best, for it does not stain so diffusely and intensely as do the other dyes. Giemsa's stain is very useful when both bacterial and cellular elements are present, such as smears from the trachea or bronchi.

All staining is hastened by heating over a flame until the fluid steams. Boiling should be avoided.

Another very useful stain is Pappenheim-Saathof methyl green. Bacteria are stained brilliant red and the nuclei of cells blue or purple. It is frequently used in staining smears for gonococci.

Methyl green,	0.15	grams;
Pyronin,	0.50	"
Alcohol—95 per cent.,	5.0	c.c.;
Glycerin,	20.0	"
2 per cent. carbolic acid water,	100.0	"

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[1] The saturation strengths of basic fuchsin, methylene blue and safranin in alcohol (96 per cent.) solution are 3.0, 7.0 and 4.0 per cent. respectively.

Gram's Method of Staining.—1. Cover the preparation with aniline-methyl-violet solution for one to three minutes.

2. Wash in water for two or three seconds.

3. Cover the preparation with Gram's solution of iodine for one to two minutes.

4. Wash with 95 per cent. alcohol until the color ceases to come out of the preparation.

5. Wash in water for two or three seconds.

6. Counterstain with bismarck brown (saturated aqueous solution for thirty seconds), pyronin (1 per cent. aqueous solution for thirty seconds), safranin ($\frac{1}{10}$ per cent. aqueous solution for twenty seconds) or dilute fuchsin (Ziehl's carbolfuchsin diluted with water, 1–50 or 1–100, for twenty seconds).

7. Wash in water two or three seconds, dry, and mount.

Certain bacteria retain the methyl violet after treating with the iodine solution and alcohol, and are consequently said to be Gram stained or Gram positive. Others do not retain the methyl violet and are subsequently stained by the relatively weak counterstain. These are said to be Gram decolorized or Gram negative. Bacteria when stained by it appear dark blue or black, while the nuclei of the cells are rather faintly stained or not stained at all. The method is especially useful in the demonstration of bacteria which are stained by it when they are present in small numbers or when a few Gram-staining bacteria are mixed among numbers of bacteria which do not stain by this method. It also has some value as a means of differentiating between bacteria which may be very much alike in size and shape.

In this connection it should be pointed out that bacteria which retain the stain by this method, when taken from cultures a few days old, may not be stained by it if taken from older cultures. Therefore, Gram's method, if used as a means of differentiation, should be applied only to bacteria in actively growing cultures.

In the following table the behavior of the more important pathogenic bacteria toward the method of Gram is indicated:

18

Stained by Gram's Method	Decolorized by Gram's Method
Staphylococcus pyogenes aureus.	Gonococcus.
Staphylococcus pyogenes albus.	Meningococcus.
Streptococcus.	Micrococcus catarrhalis.
Pneumococcus.	Typhoid bacillus.
Micrococcus tetragenus.	Paratyphoid bacilli.
Bacillus diphtheriæ.	Bacillus coli communis.
Bacillus tuberculosis.	Spirillum of Asiatic cholera.
Bacillus of anthrax.	Bacillus pyocyaneus.
Bacillus of tetanus.	Bacillus of influenza.
Bacillus Welchii and related anaërobic	Bacillus of glanders.
bacilli.	Bacillus proteus.
Bacillus botulinus.	Bacillus mucosus capsulatus.
	Bacillus of dysentery.
	Bacillus of bubonic plague.
	Bacillus of chancroid.

Sterling's Modification of Gram's Method.—The formula for this stain is given on page 78. The oil and water are added to the alcohol in the order named, and the resulting mixture is added slowly to the gentian violet while grinding in a mortar. The stain is much more stable than Gram's original solution, and to stain properly only requires one-half to one minute.

W. H. Smith's Method of Staining Bacteria in Sputum.— This has been found particularly useful in demonstrating the pneumococcus in the sputum. The sputum or other material should be fresh. The smears should be spread as thinly as possible and fixed by passing through the flame in the usual manner.

1. Stain in aniline-gentian-violet solution for a few seconds, gently warming until the staining fluid steams.

2. Wash in water.

3. Cover with Gram's solution of iodine for thirty seconds.

4. Wash with 95 per cent. alcohol until the color ceases to come out.

5. Wash in absolute alcohol for a few seconds.

6. Stain one to two minutes in a saturated aqueous solution of eosin.

7. Wash with absolute alcohol for a few seconds.

8. Clear with xylol.

9. Mount in balsam.

The pneumococcus is stained blue-black, while the capsule is stained pink. With the following modification it has been used by Smith as a routine stain for sputum. The advantage of this modification is that influenza bacilli and other bacteria which do not stain by Gram's method are clearly brought out, as are also eosinophilic leucocytes. This modification consists in washing the preparation with Löffler's alkaline methylene-blue solution just after it has been stained with eosin, as described above, and then, after the excess of eosin has been removed by the methylene-blue, steaming the methylene-blue solution for a few seconds while on the smear. The preparation is then washed in water, rinsed with alcohol, cleared with xylol, and mounted in balsam.

Methods for Staining Acid Fast Bacilli.—See page 393.

Methods for Staining Polar Bodies.—See page 349.

The Staining of Spores.—Spores take up the aniline dyes with difficulty, probably owing to their dense protective envelope. When once stained, however, they do not give up their color easily, and resist decolorizing agents. The smear preparations should be thinly spread.

Abbott's Method.—1. Stain the smear preparation deeply with methylene-blue, heating repeatedly until the staining solution boils, but do not boil continuously, for about one minute.

2. Wash in water.

3. Wash in 95 per cent. alcohol containing 0.2 to 0.3 per cent. hydrochloric acid.

4. Wash in water.

5. Stain for eight to ten seconds in aniline-fuchsin solution.

6. Wash in water.

The spores are stained blue and the bodies of the bacteria red.

Moeller's Method.—1. Wash the smear preparation in chloroform for two minutes.

2. Wash in water.

3. Treat with 5 per cent. solution of chromic acid one-half to two minutes.

4. Wash in water.

5. Stain with carbol-fuchsin, heating slowly until the fluid boils.

6. Decolorize well in a 5 per cent. solution of sulphuric acid.

7. Wash in water.

8. Stain in aqueous solution of methylene-blue (1 gram to 100 c.c.) thirty seconds. The spores will be red, the bodies of the bacteria blue.

The preliminary treatment with chloroform is to cleanse the preparation.

Fränkel's Modification of Moeller's Method.—Use as a mordant either equal parts 10 per cent. potassium bichromate and 5 per cent. chromic acid, a 5 per cent. solution of carbolic acid, or a 20 per cent. solution of tannin. The mordants keep indefinitely.

1. Cover fixed smear preparations with *one* of the mordants and heat until the stain boils. Repeat two or three times.

2. Wash with water.

3. Cover with carbol fuchsin and heat until the stain boils. Maintain this temperature for one or two minutes.

4. Decolorize with 5 per cent. sulphuric acid.

5. Wash with water.

6. Counterstain with diluted aqueous methylene-blue.

Pouring the mordant and carbol fuchsin both on a bit of filter paper covering the smear prevents spattering on boiling.

Fiocca suggests the following rapid method: "About 20 c.c. of a 10 per cent. solution of ammonia are poured into a watch-glass, and 10 to 20 drops of a saturated aqueous solution of gentian-violet, fuchsin, methylene-blue, or safranin added. The solution is warmed until vapor begins to rise, then is ready for use. A very thinly spread cover-glass, carefully dried and fixed, is immersed for three to five minutes (sometimes ten to twenty minutes), washed in water, washed momentarily in a 20 per cent. solution of nitric or sulphuric acid, washed again in water, then counterstained with a watery solution of vesuvin, chrysoidin, methylene-blue, malachite-green or

safranin, according to the color of the preceding stain." This whole process is said to take only from eight to ten minutes, and to give remarkably clear and beautiful pictures.

The Staining of Flagella.—All motile bacteria are provided with delicate wavy, hair-like prolongations of their protoplasm, called flagella, which are of comparatively great length. These flagella are the locomotor organs of the organism. The number of them attached to each individual varies to a considerable extent with the species of the bacteria. Thus the individuals of some species have but one flagellum, while the individuals of other species may have few or many springing from all parts of the organism.

The flagella are not rendered visible by the ordinary methods of staining, but special methods are necessary for their demonstration. These methods depend essentially upon the use of a mordant, which causes the flagella to take up the stain.

The cover-glasses must be absolutely free from grease in these methods, so that the watery fluids may be spread evenly over them and not run into patches. The cover-glasses may be prepared by warming them in concentrated sulphuric acid for a time, washing them in water, and keeping them in a mixture of equal parts of alcohol and strong ammonium hydroxid solution.

When used they are to be dried on a cloth which has previously been soaked in ether and allowed to dry, in order that it may contain no trace of fat. Another way to treat the cover-glasses is to take them from alcohol, dry them with a clean cloth, and then heat them by means of the cover-glass forceps in the Bunsen flame to burn off any fat or grease.

The bacteria must be distributed upon the cover-glass well separated from one another in these methods. They should not be subjected to too much manipulation in doing this, for the flagella are readily broken off. A good way is to make a dilute suspension of the bacteria in distilled water, and place one or two loopfuls of this on the cover-glass, not spreading with the loop, but making the suspension flow over the surface by inclining the cover-glass.

Another way is to place two drops of water on a cover-glass and to draw the infected wire once through one of them across the surface, and then once through the other drop, thus making two streaks. This subjects the bacteria to less manipulation and gives a good distribution in places.

The cover-glasses prepared as above indicated are to be allowed to dry in the air, and are then to be heated for a few seconds over a flame while held between the fingers. They are then ready to be stained by any of the methods given below. The cultures used for the preparations should not be older than eighteen to twenty-four hours. Solid culture-media, such as agar, should be employed.

Löffler's Method.—Treat the preparation for about one minute with the freshly filtered mordant solution, which is—

Aqueous solution of tannic acid (20 grams tannic acid
    to 100 c.c. water),   10 c.c.;
Cold saturated solution of ferrous sulphate,   5 "
Saturated aqueous or alcoholic solution of gentian-
    violet or fuchsin,   1 "

The cover-glass is to be covered with this while held with the cover-glass forceps, as in ordinary methods of staining. The mordant, thus placed on the cover-glass, may be gently heated by holding the preparation high over the flame for a period of about one minute, but it must not be boiled. After this the preparation is to be washed in water, and then stained with a freshly prepared and filtered solution of aniline-gentian-violet or aniline-fuchsin, with gentle heating for thirty to sixty seconds. It is then again washed in water, and mounted in water or balsam for examination.

In using this method, as well as others, an important thing to avoid is overheating. The mordant may be freshly mixed every time or kept indefinitely for use.

The ferrous sulphate solution should always be freshly prepared, for it rapidly decomposes. The solution of tannic acid keeps well, however.

The addition of varying quantities of acids or alkalies for different species of bacteria, as recommended by Löffler, is not necessary.

Williams' Method.—This is a modification of van Ermengem's method along the lines of the modification of Hinterberger and others. It has been adopted by Dr. Hugh Williams after a large experience with various methods in the Laboratory of the Massachusetts General Hospital.

The method is capable of giving black bacteria and flagella, with little or no precipitate. The method is as follows:

1. Prepare a mordant consisting of

Alumnol,[1] 1 per cent. solution,	5	c.c.;
Osmic acid, 2 per cent. solution,	5	"
Tannin, 20 per cent. solution,	15	"

Shake the mixture, and add three drops of glacial acetic acid, and again shake.

2. Apply the mordant less than one minute without heating. Wash thoroughly in water.

3. Cover the preparation, for about one minute, with a 1 per cent. solution of silver nitrate to which sufficient ammonium hydroxid has been added to keep the silver in solution.

4. Wash in water.

5. Wash with 0.6 per cent. solution of sodium chlorid.

6. Flood the preparation with a 30 per cent. solution of ammonium hydroxid, and immediately wash in water.

7. Apply a few drops of Ortol photographic developer. The directions for making up this developer come with the Ortol.

8. Wash in water.

9. Cover with a 1 per cent. solution of gold chlorid for a few seconds.

10. Wash in water, and apply Ortol developer for a few seconds.

11. Wash in water, and cover with a 1 per cent. solution of mercuric chlorid for a few seconds.

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[1] Farbwerke vorm. Meister Lucius u. Brüning, Höchst a. M., Germany.

12. Wash in water.

13. Apply Ortol developer for a few seconds.

14. Wash in water, and repeat the application of chlorid of gold, the washing, and the application of the developer two or more times. Between the various applications of the chlorid of gold the preparation should be inspected with a high, dry lens to determine the progress of the staining. This is readily done by placing the cover-glass, charged side upward, on a slide. In this way the process of impregnation with gold may be controlled, for the flagella, if stained, may be easily seen with the high-power dry lens.

The preparation is very conveniently held during the process in cover-glass forceps. The washing is best done in a small stream of water from a faucet. The various solutions are conveniently applied from dropping-bottles.

It will be seen that the process consists essentially in the impregnation of the flagella with silver, followed by intensification, in the photographic sense, with mercury and gold. The object of the application of the sodium chlorid and ammonia is to remove the excess of silver compounds which adhere to the surface of the cover-glass in spite of washing. This excess of silver compounds is chiefly responsible for the precipitates which appear on the preparation after the intensification. In spite of the application of the sodium chlorid and ammonia solutions, some precipitate will occur if the intensification is pushed too far. On this account it is advisable to observe the progress of the intensification under the microscope as above indicated.

Although this method may appear complicated, in practice it requires but a few minutes to stain a preparation.

Claudius' Method for Staining Flagella.—1. Fix the cover-glass or slide preparation by heating in a drying oven or hot air sterilizer to 110° C. in such a manner that this temperature is reached slowly in the course of half an hour. During the heating the preparation is to be enclosed in dry filter-paper.

2. Cover the preparation with, or immerse it in, the following mixture for twenty to thirty minutes at room temperature:

1 per cent. aqueous solution of chromic
    acid,   20 c.c.;

Freshly prepared aniline water,   0.5 "

Glacial acetic acid,   10   drops.

The aniline water is prepared by shaking together for a few minutes 1 part of aniline oil and 25 parts of distilled water and filtering through wet filter-paper.

3. Wash in water and dry in air.

4. Mount in balsam or cedar oil and examine with strong light and open condenser.
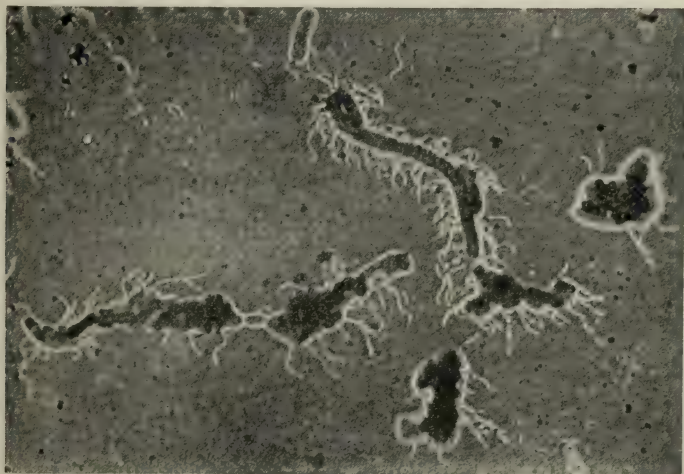
Fig. 27.—Typhoid bacilli showing flagella, after Claudius' method; × 1500 (Wright and Brown).

The method depends on the formation of aniline black in the mixture and the deposit of a bluish film on the preparation in which the flagella stand out uncolored.

Any film formed on the uncharged surface of the preparation may be removed with a towel wet with aniline oil.

Zettnow's Flagella Staining Method.—Solution 1: Dissolve 2 grams of tartar emetic in 40 c.c. of water. Solution II: Dissolve 10 grams of tannin in 200 c.c. of water. To the 200 c.c. of Solution II, warmed to 50° or 60° C., add 30 c.c. of the tartar emetic solution. The turbidity of the

mordant should entirely clear up on heating. The mordant should keep for months if a small crystal of thymol is added to it.

Next dissolve 1 gram of silver sulphate in 250 c.c. of distilled water. Of this solution take 50 c.c. and add to it drop by drop ethylamine (this comes in a 33 per cent. solution) until the yellowish-brown precipitate which forms at first is entirely dissolved and the fluid is entirely clear. It requires only a few drops.

The bacterial preparations prepared as described above are floated in a little mordant contained in a Petri dish which is heated over a water-bath for five to seven minutes. Take the dish containing the preparation off the water-bath and as soon as it becomes slightly opalescent, as the result of cooling, remove the cover-glass preparation and wash thoroughly in water. Then heat a few drops of the ethylamine silver solution upon the mordanted cover preparation until it just steams and the margin appears black. Next wash thoroughly in water and mount.

This method is highly recommended by Stitt.

The Staining of Capsules of Bacteria.—*W. H. Smith's Method for Smears.*—1. Make a thin smear preparation from fresh sputum, or pneumonic, pleural, or pericardial exudate.

2. Pass through flame.

3. Cover with a 10 per cent. aqueous solution of phosphomolybdic acid (Merck) four to five seconds.

4. Wash in water.

If the micro-organism is Gram-staining, like the pneumococcus or streptococcus, proceed as follows:

5. Aniline oil methyl-violet, steaming one-quarter to one-half minute.

6. Wash in water.

7. Treat with Gram's solution of iodin, steaming one-quarter to one-half minute.

8. Decolorize with 95 per cent. alcohol.

9. Wash in water.

10. Stain with a 6 per cent. aqueous solution of eosin one-half to one minute, warming gently.

11. Wash in water.

12. Wash in absolute alcohol.

13. Clear in xylol and mount in xylol balsam.

The capsule will be found to be distinct, clear cut, eosin-stained about the Gram-stained micro-organism.

If the micro-organism is Gram-decolorizing, after Step 4 above proceed as follows:

5. Stain with a 6 per cent. aqueous solution of eosin one-half to one minute, warming gently.

6. Wash in water.

Fig. 28.—Capsulated micrococci in a cover-glass preparation from an endocarditic vegetation stained by W. H. Smith's method; × 2000 (W. H. Smith; photo by L. S. Brown).

7. Counterstain with Löffler's methylene-blue solution one-quarter to one-half minute, warming gently.

8. Wash in absolute alcohol.

9. Clear in xylol and mount in xylol balsam. The capsule will appear eosin-stained about the blue-stained microorganism.

Hiss's Method.—Dry the smear preparation in the air and fix by passing two or three times through the flame. Cover with a mixture of 5 c.c. of saturated alcoholic solution of fuchsin or methyl-violet and 95 c.c. of distilled water and warm until steam appears. Wash off the mixture with 20 per cent. aqueous solution of sulphate of copper. Dry without washing in water. The capsule is blue and the body of the bacterium purple.

Richard Muir's Method.—1. The preparation is dried and stained in filtered carbol-fuchsin for half a minute with gentle heat.

2. Wash slightly with methyl-alcohol and then well in water.

3. Place in the following mordant for a few seconds:

Saturated solution corrosive sublimate,   2 parts;
Tannic acid solution, 20 per cent.,   2   "
Saturated solution of potash alum,   5   "

4. Wash well in water.

5. Treat with methyl-alcohol for about a minute. The preparation has a pale reddish appearance.

6. Wash well in water.

7 Counterstain with a watery solution of ordinary methylene-blue for half a minute.

8. Dehydrate in alcohol, clear in xylol, and mount in balsam.

The bacteria are stained a deep crimson and the capsules a blue tint. The capsules of bacteria in cultures may sometimes be demonstrated by this method.

THE STAINING OF BACTERIA IN SECTIONS

Bacteria are demonstrated in sections of tissues almost entirely by means of the aniline dyes, of which three have thus far proved themselves to be particularly valuable, namely, methylene-blue, fuchsin, and methyl-violet. Of each of these dyes, one or more solutions have become famous because of their efficacy in staining and their keeping qualities. They will be referred to later.

All bacteria yet known will stain when placed in appropriate staining solutions. Some, however, are stained quickly, while others are stained with difficulty; some give up the stain readily to decolorizers, while others retain it tenaciously. In consequence of their reactions to certain dyes and to certain decolorizers, bacteria, from the point of view of staining, may be divided into two main groups:

1. Bacteria which do not stain by Gram;
2. Bacteria which stain by Gram.

Included in the second group is a small group of acid-fast bacilli which can be stained in a special way by what is known as the tubercle-bacillus method.

The organisms of the second group are much more easily demonstrated in tissues than those in the first group, because it is possible to stain them one color and the nuclei of the cells another color. In other words, it is possible to stain them so that they are differentiated from the tissue in which they lie, and hence stand out prominently.

The organisms of the first group have no differential stain; they take the same color as the nuclei of the tissue. Moreover, although they stain easily, most of them do not stain deeply, and readily part with the color they have taken up.

It has been customary in the past to fix all tissues in which bacteria were to be demonstrated in alcohol. Of recent years formaldehyde has been much used for the same purpose. Zenker fixation can be recommended as being superior to either because of its perfect preservation not only of all bacteria but also of the tissues, so that by means of proper staining both the pathogenic organism and the lesion it produces can be perfectly and faithfully demonstrated.

Sections which are to be stained for bacteria may be divided into two classes: paraffin sections and celloidin sections.

Paraffin sections should, as a rule, be attached to the slide by means of Mayer's glycerin-albumin mixture.

Celloidin embedding is to some extent a drawback to the stains for certain organisms, because the celloidin tends to hold the color, so that the bacteria are not so distinct as they otherwise would be. Still, it is so important to be able to stain bacteria in celloidin sections that particular care is devoted in the following pages to methods which obviate most of the difficulties.

It will usually be found advisable to attach celloidin sections to the slide by means of ether-vapor. They will then keep perfectly flat in any staining solution, and may be heated without danger of wrinkling or contracting. The heat should never be applied directly under a section, but at one end of the slide.

Pathogenic Bacteria which do not Stain by Gram (See page 274).—The staining solutions and methods employed for demonstrating this group of bacteria are applicable also to most of the organisms in the group which stain by Gram's method, and are, therefore, of great importance.

Of the bacteria which do not stain by Gram or by the tubercle bacillus method, certain ones deserve special mention on account of their frequent occurrence or on account of the difficulty of demonstrating them in tissues, and certain variations in staining methods which have proved serviceable will be given. Löffler's methylene-blue solution has in the past been generally considered the most useful stain for this class of bacteria, but better results can be obtained by two other methods. The first is by means of the eosin-methylene-blue stain after Zenker fixation, a method which can be most highly recommended not only on account of the staining of the bacteria but also for the faithful demonstration of the histological changes present in the tissues. The second method is by means of Wolbach's modification of Giemsa's stain which also gives most excellent results after Zenker fixation. Both of these staining methods are given elsewhere. (See pages 100, 437.)

Two other methods are added because they are sometimes useful.

Löffler's Methylene-blue Stain for Bacteria.—1. Stain paraffin sections twenty minutes to twenty-four hours.

2. Wash in weak acetic acid, 1:1000, for ten to twenty seconds.

3. Absolute alcohol, two or three changes, to differentiate and dehydrate (as a rule, only a few seconds are required for this step).

4. Xylol.

5. Xylol balsam.

For celloidin sections use 95 per cent. alcohol; blot, and treat with xylol; repeat until sections are clear; mount in xylol balsam.

This solution of methylene-blue is extremely useful, because it will stain all bacteria except the tubercle-bacillus group.

Other solutions which may be used in the same way are—aniline-methyl-violet, Sterling's solution of methyl-violet, a simple aqueous solution of methyl-violet, and Ziehl's carbol-fuchsin.

Methyl-green-pyronin stain (Unna-Pappenheim) as modified by Saathoff for bacteria:

Methyl-green,	0.15 gms.;
Pyronin,	0.50 "
96 per cent. alcohol,	5.00 c.c.;
Glycerin,	20.00 "
2 per cent. carbol-water ad,	100.00 "

Stain sections two to four minutes, then wash in water, dehydrate quickly in absolute alcohol, clear in xylol, and mount in balsam. If acetone is used instead of absolute alcohol for dehydration there is less danger of decoloriz ng the cystoplasm of the cells.

Pathogenic Bacteria which Stain by Gram (See page 274).—These organisms, with the exception of the tubercle-bacillus group, are all readily stained by the general methods employed for staining under Group 1. For staining most of them in sections, however, the differential Gram-Weigert method will be found to give the most satisfactory results.

The Gram Staining Method.—Directions for staining paraffin sections: 1. Stain in aniline-methyl-violet five to twenty minutes.

2. Wash in normal salt solution or water.

3. Iodin solution (1:2:300) one minute.

4. Wash in water.

5. Absolute alcohol, several changes, until no more color is given off and the section is apparently decolorized.

6. Xylol.

7. Xylol balsam.

This method is not suited for celloidin sections, because the alcohol does not decolorize the celloidin sufficiently. In fact, it is better to reserve Gram's method for cover-slip work alone, and to use instead of it, for sections of all kinds, Weigert's modification. This consists simply in the use of aniline oil

instead of alcohol as a decolorizer. The method is easily acquired, is perfectly adapted not only to paraffin but also to celloidin sections, and the results are more perfect than after Gram.

The Gram-Weigert Staining Method.—Preferably Zenker's fixation, paraffin sections:

1. Stain sections lightly in alum-hematoxylin.
2. Wash in running water.
3. One per cent. aqueous solution of eosin, one to five minutes.
4. Wash in water.
5. Aniline methyl-violet, one-half to one hour.
6. Wash off with water.
7. Lugol's solution, one to two minutes.
8. Wash off with water.
9. Blot with filter-paper and dehydrate and clear in several changes of aniline and xylol, equal parts.
10. Wash off with xylol.
11. Mount in xylol-colophonium.

Verhoeff's Modified Gram Stain for the Leptothrix of Parinaud's Conjunctivitis.—He finds it superior to the ordinary methods for staining Gram-positive bacteria and leptothrix in sections. Zenker's fixation preferred. Either celloidin or paraffin sections may be used.

1. Stain lightly in alum-hematoxylin and eosin, mount in Canada balsam.
2. After five minutes or longer (ten years is not too long) remove cover-slip, by aid of heat if necessary, and wash off excess of balsam with xylol. If celloidin section, remove from slide. Chloroform, 95 per cent. alcohol, water.
3. Sterling's gentian violet, twelve minutes.
4. Water.
5. Lugol's solution (1:2:100), twenty seconds. Water.
6. 95 per cent. alcohol, twenty seconds.
7. Chloroform, fifteen seconds.
8. Oil of origanum, fifteen seconds.
9. 95 per cent. alcohol, thirty seconds. This removes excess of stain from celloidin.

10. Oil of origanum. If celloidin section, place on slide and blot.

11. Wash off with xylol and blot.

12. Mount in xylol-balsam.

Great care is required in the differentiation in alcohol and chloroform. A variation of a few seconds here makes a great difference in the results. It is therefore well to carry a number of sections through Step 5 and then differentiate each separately, varying the time a few seconds from that stated. If the differentiation is perfect, the leptothrix filaments as well as the dots in them will be stained, otherwise only the dots may be stained, so that the organisms will appear as rows of dots. If the differentiation is carried too far, especially in the 95 per cent. alcohol (6), the organisms may be completely decolorized. In the case of Gram-positive bacteria the preliminary treatment with balsam may be omitted and the differentiation may be carried much further without danger of decolorizing the organisms.

The Staining of Capsules in Sections.—*William H. Smith's Method for Sections.*—1. Fixation in Zenker's fluid and paraffin sections.

2. Cover with anilin-methyl-violet solution for a few seconds, warming by drawing the slide through the flame two or three times.

3. Wash with Gram's iodin solution.

4. Wash with formalin (40 per cent. formaldehyde solution).

5. Decolorize with 95 per cent. alcohol.

6. Wash quickly with Gram's iodin solution.

7. Cover with a special eosin mixture (see below), warming in the flame for a few seconds.

8. Wash, dehydrate with alcohol, clear with xylol, and mount in balsam.

To obtain the best results the duration of the application of the various reagents must be varied with each preparation, and in some instances, where very deep staining is desired, the stronger solution of iodin, Lugol's solution, may give better results.

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The decolorization by alcohol may have to be supplemented by washing with ether or with aniline-xylol, for the Gram staining may be so intense as to mask the red staining capsules. This is particularly true in the case of Streptococcus viridans or streptococci in certain cases of endocarditis in which a very narrow capsule may be demonstrable by this method.
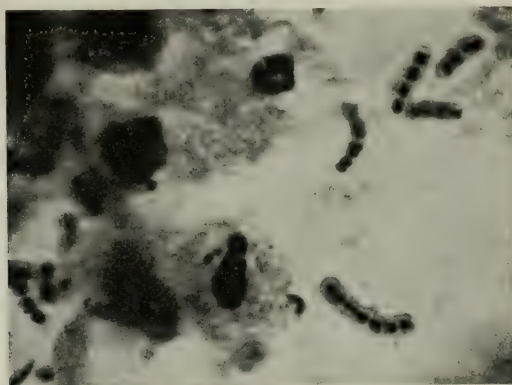
Fig. 29.—Cocci with capsules in a section of lung; × 2000 (W. H. Smith and L. S. Brown).

The special eosin mixture is made by shaking 1 part of aniline green in 200 parts of a 3 to 6 per cent. aqueous solution of yellowish water-soluble eosin and, after one or two hours' standing, filtering to remove the precipitate.

PATHOGENIC BACTERIA AND FUNGI

The number of species of bacteria of pathogenic signifi-
cance which are *commonly* encountered in pathological
processes in man is a small one. These comprise Staphylo-
coccus pyogenes aureus, the streptococci, the pneumococci,
Bacillus coli communis, the typhoid bacillus, Bacillus diph-
theriæ, and Bacillus tuberculosis. It is with infections with
these few species that the pathologist is most frequently
concerned, and the determination of the presence of these alone
comprises by far the greater part of the bacteriological work
which he is called upon to do.

In the following descriptions of the important pathogenic
bacteria which are concerned in human pathology the main
object will be to give those characteristics which will serve
for their identification, rather than an exhaustive consideration
of their various properties and modes of growth.

Staphylococcus Pyogenes Aureus.—*Morphology.*—Rather
small cocci, frequently arranged in masses or clumps.

Stained by Gram's method.

Blood-serum.—The colonies are golden yellow in color.
They are rounded, shining, slightly elevated, and may attain
a diameter of 2 mm. or more after remaining for thirty-six hours
in the incubator. The color of the colonies varies from a pale
yellow to a deep orange. Young colonies may be creamy
white, becoming yellow later.

Gelatin Stab-culture.—Growth along the line of stab, fol-
lowed by liquefaction in funnel form, with yellow sediment
and clouding of the liquefied medium (Fig. 31).

Potato.—Yellow confluent colonies.

Agar Slant.—Rather broad shining streak with sharply
defined margins, at first white in color, but later becoming
yellow.

Blood Agar.—Same as plain agar except that usually there is a zone of hemolysis surrounding the colonies. This zone is usually pink (hemoglobin present) rather than pale straw or yellow green as with streptococci.

Bouillon.—Densely clouded. A yellowish sediment is formed, and sometimes a thin pellicle is seen on the surface.

Litmus-milk.—Turned pink and coagulated.

Pathogenesis.—When inoculated into the circulation of a rabbit death follows in from eighteen hours to three days in
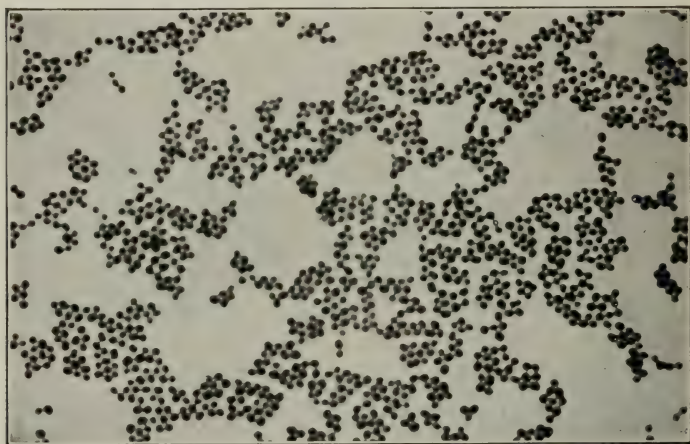
Fig. 30.—Staphylococcus pyogenes aureus from a culture; × 2000 (Wright and Brown).

the case of virulent cultures. Not all specimens of this organism are virulent. The lesions produced in the rabbit by inoculation in the ear-vein in typical cases are abscesses with infarctions in the kidneys, and miliary abscesses in the myocardium, diaphragm, and voluntary muscles. In the kidneys lines of necrosis with purulent infiltration, mainly in the pyramids, are frequently observed. This organ is the one most constantly affected. The number and extent of the lesions vary in different animals and with different cultures. They are best developed in animals which survive about three days. In animals which succumb after eighteen hours no macroscopic change may be apparent. On microscopical examination of the kid-

neys, however, small areas of necrosis will usually be found, mainly in the pyramids, surrounding masses of cocci. In the kidneys of animals which survive longer all the grades of invasion of these necrotic areas by leucocytes, up to regular abscess-formation, may be traced. By cultures the organism will be found in large numbers in the kidneys and urine of the rapidly fatal cases, and in smaller numbers in the other organs and blood of the heart.
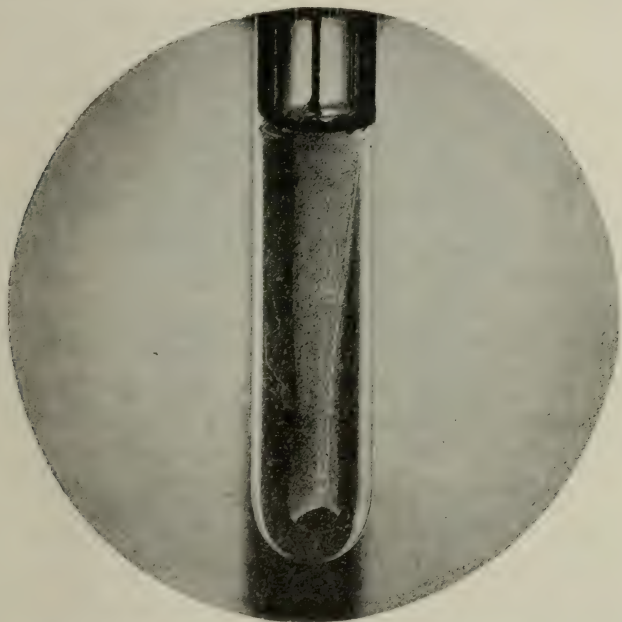
FIG. 31.—Staphylococcus pyogenes aureus: stab-culture three days old in gelatin (Fränkel and Pfeiffer).

Occurrence.—Staphylococcus pyogenes aureus is found most commonly in superficial pus-formations of a circumscribed character and also in a large number of pathological conditions, of which only the more important will be mentioned here. These are as follows: osteomyelitis, peritonitis, pleuritis, otitis media, mastoiditis, meningitis, broncho-pneumonia, and puerperal septicemia. It is quite frequently recovered in blood cultures. It may also be found in the blood of the various internal organs at autopsies in cases in which a suppurative

or other acute inflammatory process is present anywhere, with or without metastatic abscess-formation. The organism also occurs frequently in the dust of places inhabited by man, as well as on the surface of the skin and of the mucous membranes of the nose and mouth.

Diagnosis.—The Staphylococcus pyogenes aureus cannot usually be identified with any certainty by the smear examination alone. Cultures are necessary in order to differentiate from the other staphylococci and from the streptococcus.

For practical purposes the identification of the pyogenic cocci may be made by the appearance of their colonies on blood-serum and by their morphology; no secondary cultures are usually necessary,

The following staphylococci may also be present in acute inflammatory processes, but they occur less frequently than does the Staphylococcus pyogenes aureus.

Staphylococcus Pyogenes Albus and Staphylococcus Pyogenes Citreus. These organisms differ from Staphylococcus pyogenes aureus mainly in the color of their colonies. As a rule, they are much less pathogenic for rabbits than that organism.

Staphylococcus Epidermidis Albus (Welch).—"Is probably only a variety of Staphylococcus pyogenes albus. Usually grows somewhat more slowly; liquefies gelatin and coagulates milk less rapidly. Is of little virulence under ordinary conditions. Is a regular inhabitant of the epidermis, lying deeper than can be reached by disinfection of the surface of the skin" (Welch).

Staphylococcus Cereus Albus.—Very similar to Staphylococcus pyogenes albus, but does not liquefy gelatin. May occur in abscesses.

Staphylococcus Cereus Flavus.—This organism is similar to the preceding, except that it forms a lemon-yellow pigment.

Micrococcus Tetragenus.—The *colonies* are small, white, and elevated. Growth is slow.

Morphology.—Micrococci arranged in fours, or "tetrads," held together by a gelatinous substance (Fig. 32).

Stained by Gram's method. Not motile.

Gelatin Stab.—Feeble growth in the form of minute spherical masses along the line of stab with a small white, slightly

elevated point at the surface of the medium. The gelatin is not liquefied.

Agar Slant.—Moist, glistening, grayish-white translucent streak with wavy margins.

Potato.—Growth is in the form of a thick, irregular, slimy-looking patch. The growth on agar and on potato may be drawn into thin threads by the platinum wire.

Pathogenesis.—Subcutaneous inoculation of mice and guinea-pigs may lead to a fatal septicemia or only a local pus-formation.
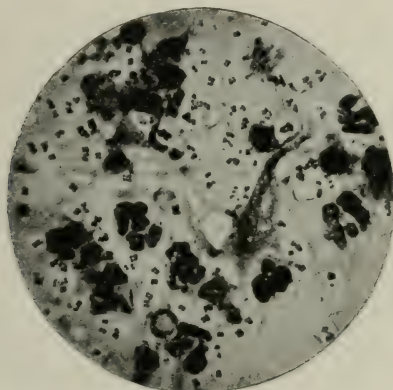
FIG. 32.—Micrococcus tetragenus in pus from a white mouse; × 615 (Heim).

Intravenous or intraperitoneal inoculation of rabbits may also produce septicemia and death.

At autopsy the micrococci, arranged in tetrads, are found in the blood generally, but most numerously in the spleen. They can readily be demonstrated by smear preparations.

Occurrence.—"Found not infrequently in phthisical cavities and sputum, occasionally in association with pyogenic cocci in abscesses connected with carious teeth and about the neck and jaws and middle ear, rarely in abscesses elsewhere. It has been considered to be non-pathogenic for man, but it has been found in pure culture in closed abscesses in man, and Viquerst has proved experimentally that it is capable of causing suppuration in human beings" (Welch).

Streptococci.—*Classification.*—Numerous suggestions have been made for the classification of the various strains of streptococci—some according to morphology and others according to one or another cultural characteristic. It has been shown that serologically there is but little similarity among the various strains, and attempts to divide the strains into groups or types, such as pneumococci, have failed.

The usual general laboratory classification divides the streptococci into two groups, Streptococcus hemolyticus and
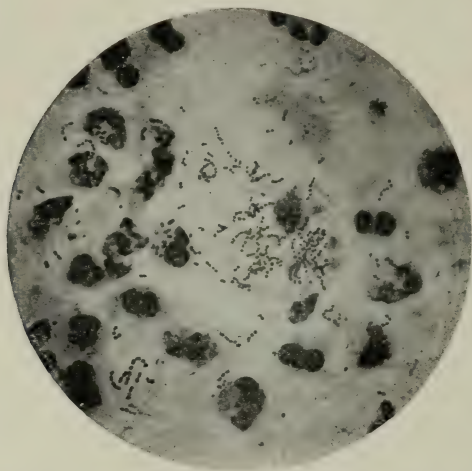
Fig. 33.—Streptococci; cover-glass preparation of the pus of an abscess; × 1000 (Fränkel and Pfeiffer).

Streptococcus viridans. Smith and Brown[1] and Brown[2] have objected to the latter name since these strains do produce true hemolysis on blood agar. They have suggested a classification based on the hemolysis produced on blood agar plates, and for the present, at least this appears to be the most satisfactory. The organisms are divided into three types depending on the hemolysis produced on blood agar plates after forty-eight hours incubation—1. *alpha*, narrow proximal zone of greenish hemolysis surrounded by an outer zone of lighter

[1] Smith and Brown, *The J. Med. Research*, 1914, xxxi, 455.
[2] Brown, *Monographs of Rockefeller Institute*, #9.

hemolysis, 2. *beta*, zone of uniform complete hemolysis, and 3. *gamma*, no hemolysis.

Morphology.—Rather small cocci arranged in chains, many of the cocci being divided into two hemispheres by a line of division running at right angles to the axis of the chain. In some strains the cocci are conical or oval in outline. The chains may be made up of many cocci and be quite long.

Smear preparations from the colonies often fail to show the characteristic chain arrangement, owing to the chains being broken up by manipulation. The chain-formation is best
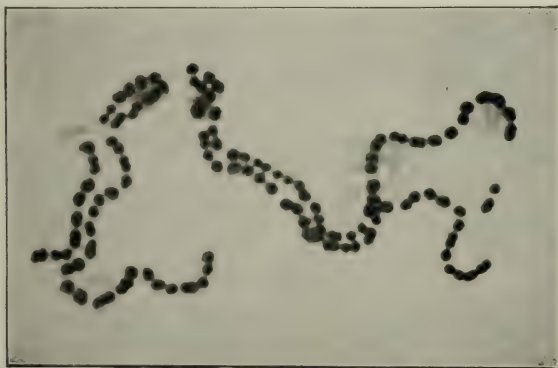
Fig. 34.—Streptococci from a culture in bouillon; × 2000 (Wright and Brown).

demonstrated in smear preparations from the "water of condensation" at the bottom of the blood-serum tube. This is essentially a bouillon culture, and it is in such fluid media that the chain-formation is best developed. In preparing the smear from this as little manipulation of the fluid as possible should be used, in order to avoid destroying the chain arrangement.

Capsules are sometimes found, as a rule with freshly isolated strains. The large and constant capsules associated with the so-called Streptococcus capsulatus or mucosus must be considered as characteristic of certain pneumococci, for this particular organism has been generally reclassified as Pneumococcus Type III in spite of occasional variants in regard to bile solubility and inulin fermentation.

Stains by Gram's method.

Blood-serum.—Minute grayish-white colonies, often looking like small grains of sand scattered over the surface of the medium. Sometimes the colonies are shining, translucent, colorless, resembling minute dewdrops.

Bouillon.—The character of the growth in bouillon is subject to considerable variation. The usual finding is a more or less flocculent growth at the bottom of the test-tube with a relatively clear supernatant fluid. Some strains produce a diffuse clouding of the medium. As a rule the latter belong to the group producing "alpha" hemolysis on blood agar, the so-called Streptococcus viridans.

Blood Agar.—The individual colonies resemble those on plain agar except that at a given incubation period the colonies are usually larger because of a more favorable culture media. The types of hemolysis produced serve as a means of differentiating as previously mentioned.

Ascitic Dextrose Bouillon.—This consists of 1 part of sterile ascitic fluid mixed under aseptic precautions with 3 or 4 parts of sterile 1 per cent. dextrose bouillon. In this medium the streptococci grow more abundantly than in ordinary bouillon.

Fig. 35.—Streptococci culture upon agar two days old (Fränkel and Pfeiffer).

Agar Slant.—Minute grayish translucent colonies (Fig. 35).

Agar Stab.—Small spherical grayish colonies along the needle-track.

Gelatin.—Growth similar to that on agar.

Litmus-milk.—Some varieties turn the medium pink and cause coagulation.

Fermentation of Carbohydrates.—Great differences exist among various strains in the ability to ferment certain carbohydrates,

such as lactose, mannite, salicin, dextrose, and saccharose. One relatively constant characteristic of streptococci is their inability to ferment inulin. This fact is made use of in the differentiation of streptococci and pneumococci. For fermentation tests it is advisable to use Hiss serum water medium.

Bile Solubility.—When four to nine parts of a suspension or bouillon culture of streptococci are added to one part of clear
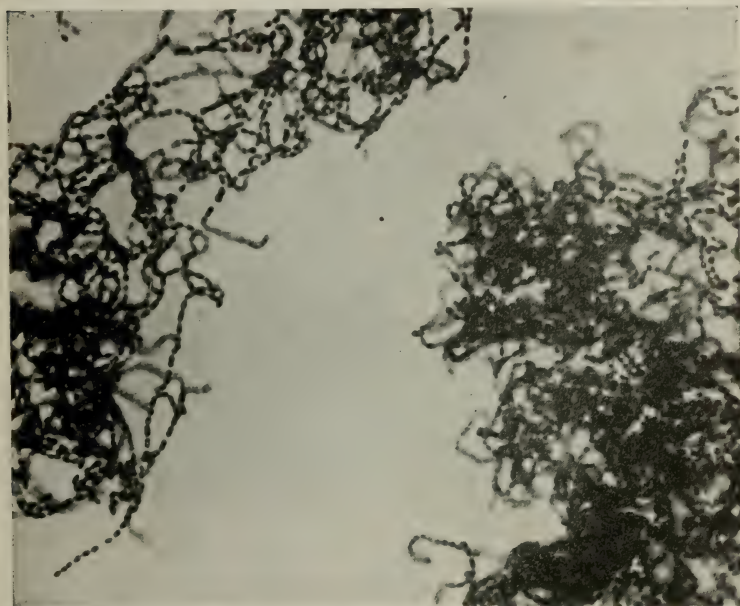
Fig. 36.—Streptococci from a culture; × 1500 (W. H. Smith and L. S. Brown).

sterile ox bile and the resulting mixture placed in a water bath at 37.5° C. for thirty to sixty minutes, no clearing of the mixture is noted. This serves as an aid in differentiating streptococci from pneumococci which are bile soluble.

Pathogenesis.—The results of the inoculation of animals are not constant, great variation in the virulence of different cultures being observed. Sometimes mice inoculated at the root of the tail or in the peritoneal cavity will die in about twenty-four hours with enlargement of the spleen and large numbers of the organism in the internal organs.

Occurrence.—The streptococcus occurs frequently in the spreading phlegmonous inflammations as well as in suppurative processes generally, and is the most common cause of septicemia. It is almost always present in inflammatory conditions of the mucous membrane of the pharynx, and is often encountered in bronchopneumonia. In erysipelas it is almost invariably the infecting organism, and it is the most frequent cause of puerperal septicemia. In many fatal cases of scarlet fever and in some cases of typhoid fever,

Fig. 37.—Streptococci in a section from a cardiac vegetation; × 1000 (W. H. Smith and L. S. Brown).

diphtheria, tuberculosis and other acute inflammatory diseases it will be found in the blood after death. It also occurs in a certain proportion of cases of peritonitis, pleuritis, meningitis, endocarditis and otitis media. In endocarditis, masses of the micro-organisms may form a large part of the substance of the cardiac vegetations. Gaining entrance to the tissues through an insignificant wound or abrasion of the skin, it may produce a rapidly fatal septicemia in a susceptible individual, in whose internal organs at autopsy large numbers of the organism will be found. This general invasion of the circulation may also be observed in cases of chronic or wasting disease, the infection occurring during the last days or hours of life (terminal infection).

Of other conditions in which it may occur, hepatic abscess, appendicitis, osteomyelitis, and synovitis may be mentioned. Although the streptococcus is distinctly one of the pus-producing bacteria, yet the inflammations of the soft parts of the extremities which are produced by it are generally characterized more by necrosis and serous or hemorrhagic exudation and infiltration than by the breaking down of tissue and frank pus-production. In this the organism is in marked contrast to the Staphylococcus pyogenes aureus, which practically always produces dissolution of tissue and pus. More-
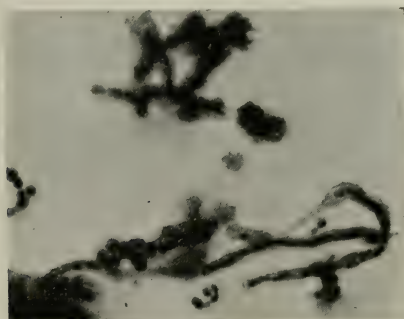
Fig. 38.—Streptococci showing capsules in a section of a cardiac vegetation; × 1500 (W. H. Smith; photo by L. S. Brown).

over, the streptococcus inflammations are more commonly accompanied by lymphangitis than are those due to the Staphylococcus pyogenes aureus.

Diagnosis.—The streptococcus may often be identified by the smear examination alone through its characteristic chain-formation, but this may not be apparent and the result of cultures must then be awaited.

Practically, the only organism with which the streptococcus may be confounded is the pneumococcus, which also grows in minute colonies and sometimes in chains. The streptococcus differs from the pneumococcus in the morphology of the individual organisms, the streptococci usually appearing as pairs of spheres, and the pneumococci as pairs of oval, conical, or lancet-shaped organisms, the broader ends of which are in apposition. Flocculent bottom growth and

clear supernatant fluid in bouillon cultures suggest streptococci rather than pneumococci. Pneumococci never cause hemolysis of the *beta* type. The usual differential tests depend upon the facts that the streptococcus is not soluble in bile and that it does not ferment inulin.

In attempts to cultivate the streptococcus it should be borne in mind that the organism grows most readily in enriched bouillon (ascitic fluid, serum or blood) containing 0.5–1.0 per cent. of dextrose. At the same time the organism dies more rapidly in media containing dextrose because of the acidity produced by the fermentation of the sugar.

The majority of streptococci occurring in pathological lesions belong to the *beta* type, Streptococcus hemolyticus. Organisms of the *alpha* type, Streptococcus viridans, are recovered chiefly from cases of endocarditis and in some instances of tonsillitis. The *gamma* type is rarely, if ever, pathogenic.

Pneumococci.—*Synonyms:* Diplococcus pneumoniæ; Micrococcus lanceolatus; Micrococcus of sputum-septicemia; Micrococcus pneumoniæ crouposæ.

From the work of Neufeld and Haendel and that of Cole and his associates in this country it appears that all pneumococci can be divided into three definite serological groups (Types I, II, and III) and a fourth group (Type IV) containing all strains which do not fall in the other three. Some attempts have been made to sub-divide the fixed types. The most noteworthy is the division of Type II into two sub-divisions— typical and atypical; the chief difference is that the typical Type II organisms are agglutinated by a much higher dilution of homologous antibacterial serum than the atypical ones.

As stated in the section on streptococci the Type III group consists of organisms formerly classified as Streptococcus capsulatus or mucosus. The fact that the majority of strains are bile soluble and ferment inulin justifies the reclassification.

Morphology.—Pairs of rather small oval, conical, or lancet-shaped organisms, the broader ends being in apposition. The organism varies somewhat in size, and one of the "pair" may be smaller than the other (Fig. 39). In some cases

atypical or involution forms are seen, especially if the culture is more than twenty-four hours old. No capsules are ordinarily observed in cultures with ordinary methods of staining.
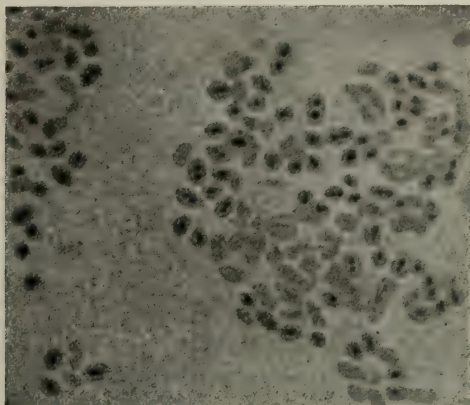
FIG. 39.—Pneumococci with capsules in a smear preparation from pericardial exudate stained by W. H. Smith's method; × 1500 (W. H. Smith; photo. by L. S. Brown).

In the "water of condensation" of the blood-serum tube, chains may be formed resembling those of the streptococcus, but differing from the chains of that organism by the oval or lancet form of the elements of which they are composed.
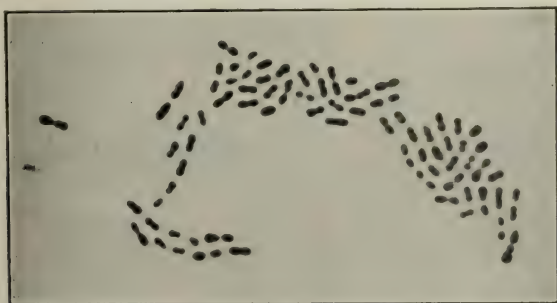
FIG. 40.—Pneumococci from a culture; × 2000 (Wright and Brown).

In exudates and in the animal body the organism is frequently encapsulated (see Fig. 39, 41). This is composed of a mucin-like substance. It may be seen usually in smear preparations

stained by the ordinary methods, especially if the preparations are examined in water-mounting.

The Type III pneumococcus morphologically resembles streptococci. The individual organisms are spherical or hemispherical and chains of varying length are almost invariably present. Whereas capsules are not commonly observed with the other types, here the opposite is true—capsules are not only easily demonstrated on isolation but persist on subculture (see Figs. 42, 46).

Stained by Gram's method. Not motile.

Bouillon.—Clouded faintly and uniformly. Growth is greatly enhanced by the addition of enriching material (ascitic fluid, serum, or blood) or 0.1–1 per cent. dextrose.

Plain Agar Slant.—Fine discrete translucent grayish colonies resembling those of streptococci except that there is a tendency to be flat.

Blood Agar Slant or Plate.—Colonies somewhat larger than those on plain agar. The Type III colonies are much larger than those of other types, are flatter, and usually coalesce. Surrounding the colonies of all types are zones of greenish hemolysis similar to those produced by the alpha type of streptococci.

Blood Serum.—Minute colorless transparent colonies (Fig. 43). Type III colonies are large, flat, colorless, and mucoid (see Fig. 44).

Litmus Milk.—Usually turned pink and coagulated. This reaction is not constant.

Fermentation of Carbohydrates.—The fermentation of inulin contained in Hiss serum water media is characteristic of pneumococci *with few exceptions* (*Type III*) and serves to differentiate them from streptococci.

Bile Solubility.—On adding 1 part of sterile ox bile or of a 10 per cent. solution of sodium taurochlorate in physiological salt solution to 4 to 9 parts of a fresh broth culture of pneumococci the mixture becomes clear. Pneumococci are bile soluble. This solution is hastened by placing the tube in a water bath at 37.5° C. for one-half to one hour.

Pathogenesis.—The pneumococcus is very pathogenic for mice and rabbits, less so for guinea-pigs.

Subcutaneous inoculation with virulent cultures causes the death of mice in from twenty-four to thirty-six hours, and of rabbits in from thirty-six to forty-eight hours, with septicemia.
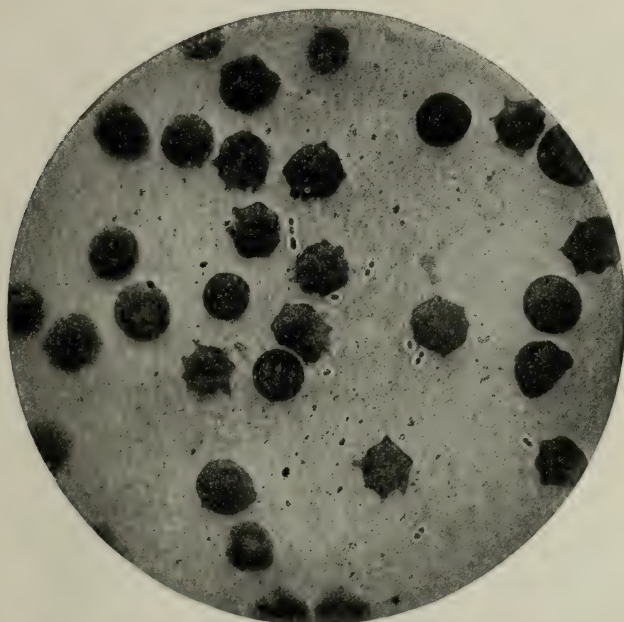
FIG. 41.—Pneumococci; smear preparation from the heart's blood of a rabbit; × 1000 (Fränkel and Pfeiffer).

This infection is the "sputum-septicemia" of Sternberg. At autopsy there will be found in the blood the characteristic encapsulated lancet-shaped organisms, usually in pairs (Fig. 41). Great variation in the virulence of the organism is observed. In some cases no effect will be produced by the inoculation; in others a more or less extensive fibrino-purulent exudation will be produced about the point of inoculation, and the animal will survive for a considerable length of time or recover. Inoculation into the ear-vein or peritoneal cavity of a rabbit will sometimes cause a rapidly fatal septicemia, when subcutaneous inoculation with the same culture will

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only cause a local reaction. The virulence of the pneumococcus is quickly lessened by cultivation.

Occurrence.—The pneumococcus may be demonstrated in the pulmonary exudate of practically all cases of genuine lobar or croupous pneumonia. Blood cultures from active cases of lobar pneumonia should give positive results in about
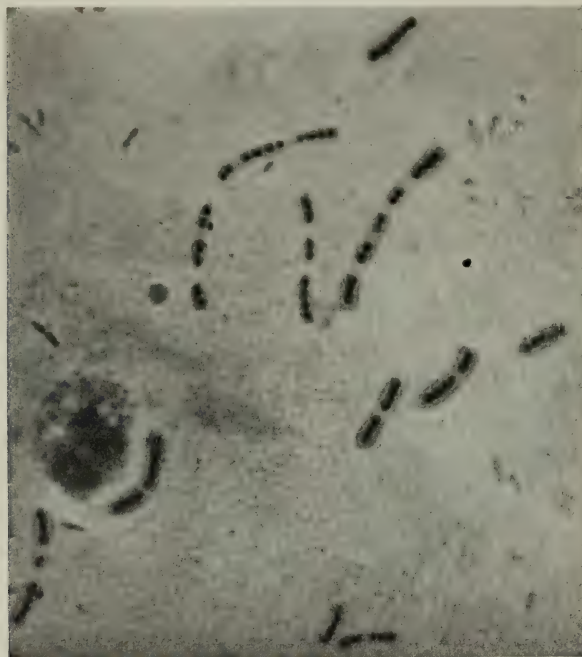
FIG. 42.—Pneumococci (Type III) in smear preparation from sputum stained by W. H. Smith's method (W. H. Smith; photo. by L. S. Brown).

20 to 40 per cent. At autopsies on cases of this disease it may be found in large numbers in the consolidated lung, and sometimes in smaller numbers in the blood. Cultures from the lung may sometimes show the presence of other bacteria in addition to the pneumococcus, but these are to be regarded as either secondary infections or contaminations from the smaller bronchi.

The pneumococci in the pneumonic exudate die in large numbers after a time, and in cases near resolution numerous

capsules may be found in smear preparations from the lung in which it is impossible to demonstrate the organism by staining methods.

The pneumococcus is also frequently found in broncho-pneumonia, acute peri- and endo-carditis, acute pleuritis and empyema, acute purulent meningitis, and in otitis media. In cases of pneumonia and bronchitis it may be present in the sputum in large numbers. It has been observed in cases of peritonitis, of synovitis, of osteomyelitis, and of abscess-formation in various situations.

Certain types of pneumococci are normally common habitants of the mouth, nose, and throat. Thus according to Avery, Chickering, Cole and Dochez[1] the incidence of Type IV is 53 per cent., Type III 28 per cent., and Type II (atypical) 18 per cent. Types I and II are seldom found except in individuals who have been in close contact with a Type I or II lobar pneumonia. The relative frequency of the various types in the mouth corresponds closely to their recovery from the lungs in cases of bronchopneumonia.

Diagnosis.—If the pneumococcus is present in very small numbers in pathological material, the quickest and most certain method of demonstrating its presence is the inoculation of a mouse with some of the material. This is also the best way to prove the identity of the organism.

Plain bouillon must be accurately titrated so that the final reaction is pH 7.6–7.8 in order to obtain good growth of the pneumococcus. If the reaction varies more than $\pm$pH 0.2 little, if any, growth will be obtained. Growth is promoted by the addition of enriching substances such as blood, serum, or ascitic fluid. The addition of dextrose up to 1 per cent. facilitates growth; at the end of twenty-four hours, although there is dense clouding of the medium due to the presence of many organisms subcultures usually show no growth. In liquid media containing 1 per cent. dextrose the acidity is markedly increased by the fermentation of the dextrose, and usually within twenty-four hours the reaction becomes such ($\pm$pH 5.1)

[1] Avery, Chickering, Cole and Dochez, *Monographs of Rockefeller Institute, #7.*

that the pneumococci are killed.   This increase in acidity can be prevented by the addition of powdered calcium carbonate to the medium.

The pneumococcus can usually be identified in exudates, blood, tissues, or sputum by examination of smear preparations alone, by reason of its peculiar morphology and its possession of a capsule.   The capsule can be seen in most instances in smear preparations, stained in the usual manner.   The capsules appear as a hyaline material usually with definite outlines surrounding the paired organisms.   It may be distinguished from the streptococcus by two characteristics, namely, that it is dissolved by bile and that it ferments inulin.

The Determination of Types of Pneumococci.—Researches carried on in the Rockefeller Institute for Medical Research[1] have shown that several types of the pneumococcus may be differentiated as the infectious agent in lobar pneumonia by agglutinative reactions with immune sera.   The technique may be described as follows:

The sputum should consist of material actually coughed up. A promising portion is selected and a bit smeared, fixed and stained by the Gram method.   The remainder is washed three or four times in sterile half Petri dishes containing sterile salt solution or bouillon.   It is then either drawn up directly through a needle into a syringe, or, if this procedure fails, is first ground up in a small sterile mortar with sterile salt solution or bouillon.   One cubic centimeter is injected into the peritoneal cavity of a mouse.   White mice are preferable, but the ordinary gray or brown mouse will answer the purpose. The mouse should be autopsied as soon as it dies.   If very sick it may be killed with ether or chloroform.

The peritoneal cavity is opened and washed out, under sterile precautions, with 4 or 5 c.c. of salt solution with the aid of a pipette provided with a rubber bulb.   The washings are immediately transferred to a sterile tube and centrifuged at low speed for a short time to throw down cells and fibrin;

[1] Avery, Chickering, Cole and Dochez, Monographs of Rockefeller Institute, #7.

then the supernatant fluid is drawn off into another tube and centrifugated at high speed until the organisms are deposited as a sediment. From this sediment, after removal of the supernatant fluid, a suspension in salt solution is made of about the same concentration as that of a bouillon culture of pneumococcus. The supernatant fluid is sometimes used for testing for the presence of soluble specific substances (precipitin reaction). For general laboratory work the supernatant fluid after the first (slow) centrifuging consisting of a suspension of the organisms in the diluted fluid of the exudate may be used for typing. This latter method really is a combination of the agglutinin and precipitin reactions, and results in a considerable saving of time.

Each, or all, of the above fluids may be tested for the type of pneumococcus, omitting the bile tube in the precipitin test, as follows:

A row of five small tubes is set up. In each of the first four tubes is placed 0.5 c.c. of the suspension, but in the fifth tube only 0.4 c.c. Then there is run into tube No. 1 0.5 c.c. of immune serum I, diluted 1:20; into tube No. 2 immune serum II, undiluted; into tube No. 3 immune serum II, diluted 1:20; into tube No. 4, immune serum III, diluted 1:5; and into tube No. 5, 0.1 c.c. of sterile ox bile. The tubes are incubated at 37° C. in a water bath and read after an hour. Often the immune serums as obtained from the producers are of such strength that the dilutions given above are not applicable. In such cases the correct preliminary dilutions are specified on the labels of the serum containers.

The identification of Type I, II, II atypical, or III of the pneumococcus is made by the appearance of clumping or agglutination of the organisms or of flocculation in the tube containing the corresponding immune serum. With Type II there is agglutination or precipitation in both dilutions of the Type II immune serum; whereas, with Type II atypical this occurs only in the tube containing the undiluted serum dilution. If there is no agglutination or precipitation in any of the tubes and the bacteria in the bile tube are dissolved, then the

organism is identified as Type IV pneumococcus. As all strains of pneumococci are dissolved by bile and all strains of streptococci are not, the fifth tube serves to prevent the mistake of classifying a streptococcus as Type IV pneumococcus. The bile is prepared by autoclaving, filtering off the precipitate, and again autoclaving. As a substitute a 10 per cent. solution of sodium taurochlorate in physiological salt solution may be used.
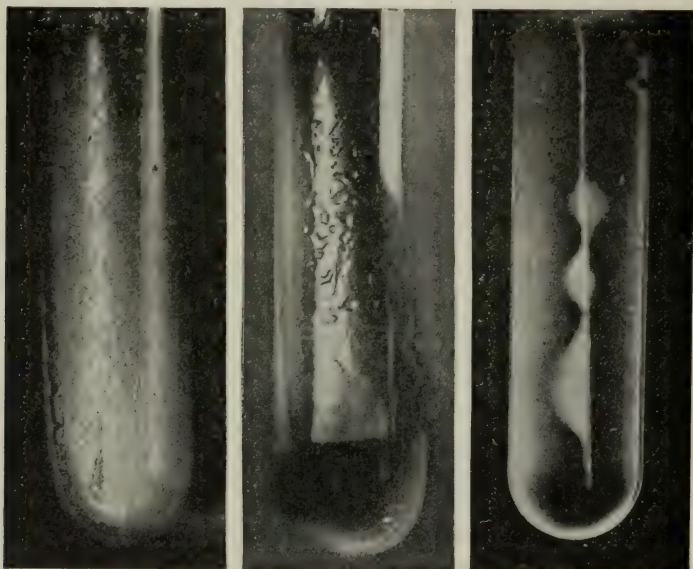
FIG. 43.   FIG. 44.   FIG. 45.

FIG. 43.—Pneumococcus; blood-serum culture.
FIG. 44.—Pneumococcus (Type III); blood-serum culture.
FIG. 45.—Pneumococcus (Type III); dextrose-agar stab culture (Oscar Richardson; photos. by L. S. Brown).

Types I and II occur in about equal proportion in more than 60 per cent. of all the cases of pneumonia investigated. Type III is the least common and is recovered in about 15 per cent. of the cases. Type IV occurs in about 20 per cent.

For control, cultures on blood-agar plates and in bouillon should be made from the heart's blood of the mouse, and with a pure bouillon culture thus obtained confirmation of the

type should be carried out as in the case of the peritoneal exudate described above.

If a positive culture from the blood of the patient be available, 10 c.c. of the fluid should be centrifugated and a suspension of the organism prepared and tested as described above. Likewise the determination of type may be made for pneumococci obtained by culture from spinal fluids and other material.
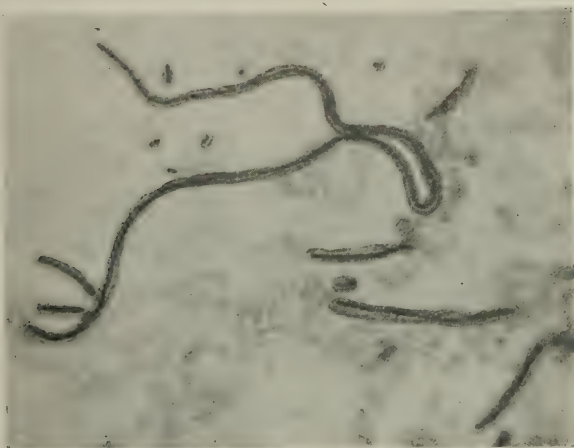
Fig. 46.—Pneumococcus (Type III), from a blood culture in a case of pneumonia; stained by W. H. Smith's method; × about 700 (W. H. Smith; photo. by L. S. Brown).

Avery[1] has devised a method for determining pneumococcus types which may be used in instances where mice are not available. The method is based upon the rapidity of growth of pneumococci in 1 per cent. dextrose blood bouillon. Great care must be taken in the selection and washing of the sputum specimen. One-half or one cubic centimeter of a bouillon emulsion of sputum is added to 4 c.c. of the culture media contained in a small centrifuge tube (15 c.c.).[2] After four hours incubation the tube is centrifuged a short time at low speed to

[1] Avery, O. T., *J. A. M. A.*, 1918, lxx, 17.
[2] The media is made as follows: To 90 c.c. bouillon (sterilized at 100° C.) add 5 c.c. each of sterile 20 per cent. dextrose solution and defibrinated rabbit's blood. The mixture is distributed in 4 c.c. amounts to 15 c.c. centrifuge tubes.

throw down the red blood cells. The supernatant fluid which is pipetted away is tested in the usual manner as described above. Smears should be stained and examined and subcultures made to blood agar plates for confirmation.

In a certain number of cases a type determination can be made from the sputum and from the urine. Both require but a relatively short time. The former method was devised by Krumwiede and Valentine[1] and the latter by workers at the Rockefeller Institute.[2]

Gonococcus.—*Morphology.*—Cocci of medium size, composed usually of two hemispheres separated by a narrow
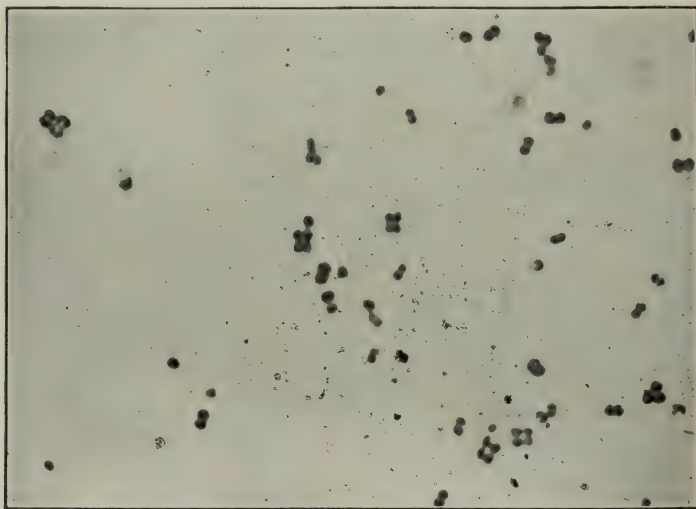
FIG. 47.—Gonococci from a culture, showing formation of tetrads and variability in the size of the cocci; × 2000 (Wright and Brown).

unstained interval. Sometimes two of these pairs of hemispheres are joined together in the manner of "tetrads," or groups of four, showing evidence that division occurs in two directions at right angles to each other (Fig. 47). Decolorized by Gram's method.

[1] Krumwiede and Valentine, *J. A. M. A.*, 1917, lxx, 513.
[2] Avery Chickering, Cole and Dochez, Monographs of Rockefeller Institute, #7.

The gonococcus will not grow satisfactorily upon any of the culture-media ordinarily employed, but requires special media for its cultivation.

The *colonies* on suitable culture-media appear after eighteen to twenty-four hours as minute, grayish, translucent points. Later they may attain a diameter of 2 mm. Under low magnifying power a well-developed colony is seen to consist of a generally circular expansion, with thin, translucent, sharply defined margins, becoming brownish, granular, and denser toward the center, which is made up of coarse brownish clumps closely packed together (Fig. 48).
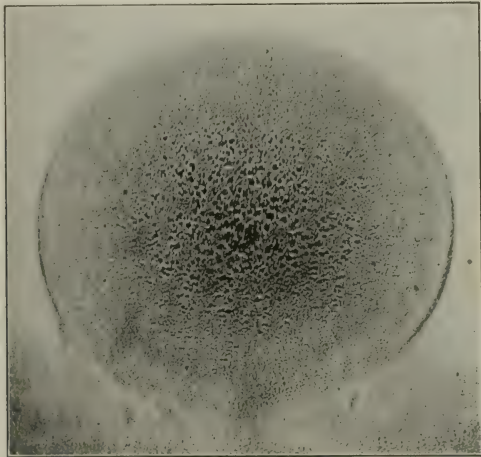
Fig. 48.—Gonococcus colony (low magnifying power; photo. by L. S. Brown).

Special Culture-media.—The essential constituent of culture-media upon which the gonococcus will grow seems to be blood-serum or similar albuminous fluid from the animal body.

Probably the most convenient culture-medium for the cultivation of the gonococcus is *hydrocele-fluid agar*. This medium consists of sterile hydrocele fluid mixed with fluid infusion agar or "hormone" agar at a temperature of 40° C., in the proportion of 1 part of hydrocele fluid to 2 or 3 parts of agar. The hydrocele fluid is to be obtained under the strictest aseptic precautions to avoid contamination with bacteria, thoroughly sterilized vessels, etc., being used. To the fluid

agar in each tube the sterile hydrocele fluid is then added in the proportion of one-third to one-half the volume of the agar, care being taken to avoid contamination. For the transfer of the serum to the agar tubes a sterilized pipette should be used. The tubes may then be poured into sterilized Petri dishes, or the tubes may be placed on their sides in a slightly inclined position and the agar allowed to solidify, thus forming "slants" which may be kept on hand ready for use. In order to test for the presence of contaminating bacteria it is well to place the plates or tubes in the incubator for twenty-four hours after they have become solid, so that any bacteria which may be present in them will form colonies and manifest themselves. Other pathological fluids which are rich in albumin, such as the serous exudate of pleuritis, may be used in the place of the hydrocele fluid as above described.

Occurrence.—The presence of the gonococcus may be demonstrated in the pus of acute gonorrhea and gonorrheal ophthalmia. It occurs also in a certain proportion of cases of purulent salpingitis. It has been found in peritonitis, endocarditis, pericarditis, myocarditis, pleuritis, and arthritis, as well as in peri-urethral abscess, in abscess of the glands of Bartholini, and in other acute inflammatory processes. In a few cases of endocarditis it has been demonstrated in the blood during life.

Diagnosis.—For practical purposes the gonococcus may be sufficiently identified in urethral pus by smear examination of the same. Cocci in the form of paired hemispheres situated *within* the pus-cells and decolorizing by Gram's method of staining may be regarded as gonococci with a fair degree of certainty.[1] The fact that they decolorize by Gram's method serves to distinguish them from the pyogenic staphylococci and streptococci, for these may also be present inside leucocytes, and may in some instances look like gonococci. The identification by this means is not beyond question. To make it more

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[1] There is no trustworthy evidence that any other Gram-decolorizing micrococcus than the gonococcus ever occurs in gonorrheal pus. F. T. Lord, working in the laboratory of the Massachusetts General Hospital, examined by cultures the pus from 22 cases of gonorrhea, and in none could he find any Gram decolorizing micrococcus other than the gonococcus.

certain the isolation and study of the suspected cocci in cultures are necessary. In cultures, not only should the organism show the peculiarities of morphology, of staining, and of colony growth above described, but it should be rigidly determined that it does not grow on ordinary agar.

In making transplants to plain agar avoid carrying over any of the albuminous material of the special culture-medium, for this material may permit some growth of the gonococcus on the plain agar.
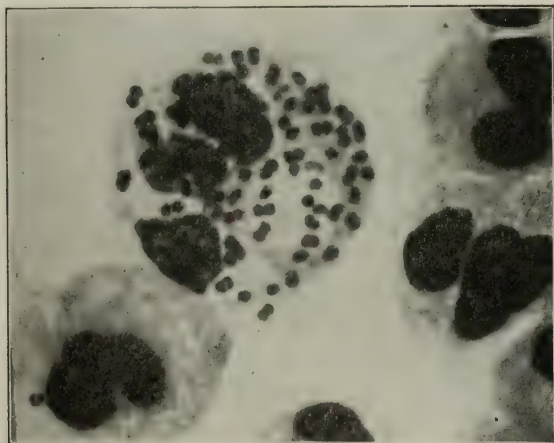
Fig. 49.—Gonococci inside a leucocyte. Smear preparation from gonorrheal pus; × 2000 (Wright and Brown).

In applying the test of decolorization by Gram's method, colonies not more than forty-eight hours old should be used, because Gram-staining cocci in older cultures may be more or less decolorized by this method.

Sugar fermentations are often useful in differentiating the gonococcus from other Gram decolorizing diplococci. Dextrose is fermented, whereas maltose, levulose, saccharose, lactose, and galactose are not fermented. These tests should be carried out in Hiss serum water media containing 1 per cent. of the various pure sugars.

If it is desired to obtain cultures of the gonococcus from the pus of gonorrheal urethritis, the case should not be more than of a few days' duration, because cases of longer dura-

tion will usually show the presence of other bacteria whose colonies overgrow the feebly growing colonies of the gonococcus. An organism which may be mistaken for the gonococcus is a coccus growing in large milk-white colonies on all media, but staining by Gram's method. This coccus is frequently found in gonorrheal pus after the discharge has lasted several days. Other cocci also occur.

The pus for culture purposes may be collected on a "swab," and the special culture-medium directly infected with this. The gonococcus retains its vitality in the pus on the swab for a number of hours, but care should be taken to avoid drying.

In a certain proportion of cases of purulent inflammation of the oviducts gonococci may be found and cultivated, as above indicated. The majority of cases, however, will have sterile pus, while in a small percentage of cases the ordinary pyogenic cocci will be present.

Cultures from the blood in cases of suspected gonorrheal endocarditis should be made in hydrocele bouillon. This consists of 1 part of sterile hydrocele fluid and 3 parts of sterile bouillon mixed under aseptic precautions.

Special Method of Staining for Gonococci (*Pappenheim, Saathoff modification*).—1. Stain smear preparation for three to five minutes in the following mixture:

Methyl-green	0.15 gms.;
Pyronin	0.50 "
Alcohol (95 per cent.)	5.00 c.c.;
Glycerin	20.00 "
Carbolic acid water (2 per cent.) ad	100.00 "

2. Wash off in water; dry; mount in xylol balsam.

Nuclei green; cocci bright red.

To Stain the Gonococcus in Sections.—The general stains used for Gram-negative bacteria give good results. After Zenker's fixation Mallory's eosin and methylene-blue method is recommended.

Micrococcus Catarrhalis.—This micrococcus may be found in the sputum in inflammatory conditions of the respira-
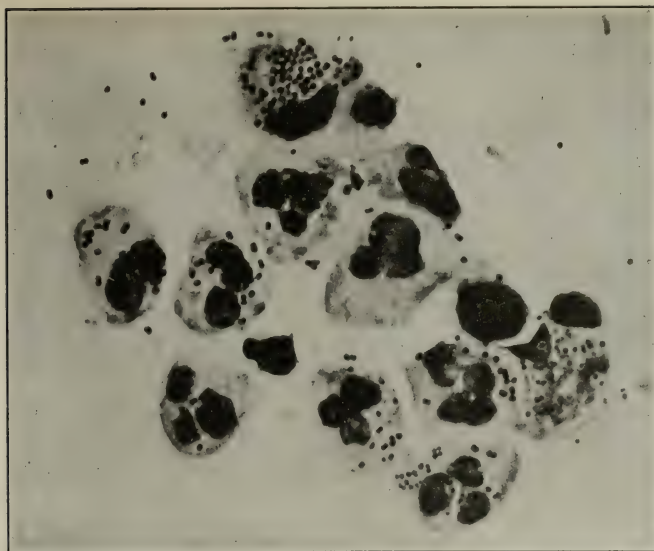
Fig. 50.—Micrococcus catarrhalis in smear from sputum (F. T. Lord; photo. by L. S. Brown).

tory tract and cannot be distinguished in its morphology and staining reactions from the gonococcus or from the meningococcus (Fig. 52). Occasional cases of meningitis have been reported in which Micrococcus catarrhalis was the causative organism. The appearances of its colonies on ordinary culture-media are, however, characteristic. They are large, white, of irregular outline, and have elevated central portions. They are friable, not viscid, and grow readily at room-temperature (Fig. 51). Two other methods for differentiation are agglutination reactions and sugar fermentations. Micrococcus catarrhalis does not ferment dextrose, maltose, levulose, saccharose, lactose, or galactose.

Meningococcus.—*Synonym:* Diplococcus intracellularis meningitidis.
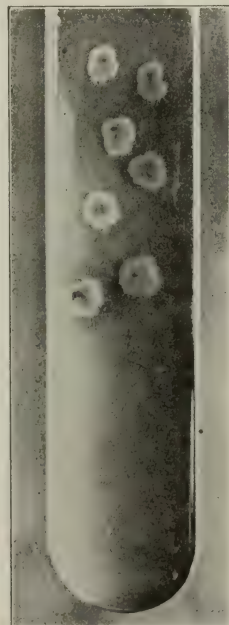
Fig. 51.—Micrococcus catarrhalis colonies on agar (F. T. Lord; photo. by L. S. Brown).

Morphology.—Diplococci, occurring as paired hemispheres, separated by a well-marked unstained interval and showing
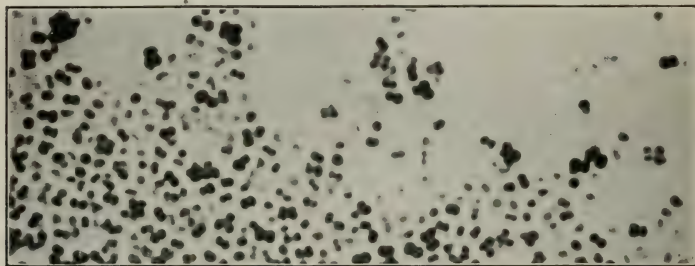
Fig. 52.—Meningococcus from a culture, showing the tendency toward grouping in fours or tetrads; × 2000 (Wright and Brown).

considerable variation in size and staining intensity in cultures (Fig. 52). The larger forms are regarded as involution or

Fig. 53.—Meningococcus in polynuclear leucocytes of meningeal exudate (Jaeger).

degenerate forms. The organism shows a tendency to grouping in fours or tetrads.

In smear preparations from the meningeal exudate the diplococcus frequently is situated inside leucocytes (Fig. 53). The appearances are very much like those of gonorrheal pus. It is decolorized by Gram's method.

In regard to cultures in general it may be said that the meningococcus when freshly isolated, grows best on "hormone" media or enriched media, such as blood agar or blood serum. The addition of 0.5 per cent. glucose exerts a favorable influence on growth. It is extremely sensitive to temperature changes and frequently cultures from positive fresh material will give negative cultural results if the material is either held at room temperature an hour or more before planting or planted on chilled culture-media. The organism will quickly die out on artificial media unless frequent transplants are made. After cultivation on artificial media for some period of time meningococci will grow profusely on relatively simple media. Dextrose agar or liver agar serve admirably for the keeping of stock cultures. Stock cultures should be stored at incubator temperature.

Blood-serum.—The colonies appear after about twenty-four hours, and after forty-eight hours may attain a diameter of 2 or 3 mm. They are round, colorless, shining, slightly convex or flat, moist, and viscid-looking. They may become confluent.

Plain Blood and Laked Blood Agar.—Round, flat, grayish, translucent, moist, shining colonies, attaining a diameter of 2 or 3 mm. after twenty-four hours in the incubator. They may become confluent, and in a "slant" culture the growth appears as a grayish, translucent, moist, shining streak about 3 mm. in width, with smooth margins. Under a low magnifying power the colonies are homogeneous, semi-translucent, and not granular.

Bouillon.—Medium slightly to moderately clouded. At the bottom of the tube a whitish sediment, which may rise as a viscid string when the tube is shaken.

Potato.—Very feeble or doubtful growth, giving the surface of the potato a moist appearance at the most.

Litmus-milk.—Growth without visible change in the medium.

Action on Sugars.—Acid without gas is produced by growth in media containing dextrose and maltose. Levulose, saccharose, lactose, and galactose are not fermented.

Gelatin.—Feeble growth. No liquefaction.

Pathogenesis.—Intraperitoneal inoculation of guinea-pigs and rabbits gives very uncertain results. Mice are said to succumb to subcutaneous inoculation, with some invasion of the blood by the organism.
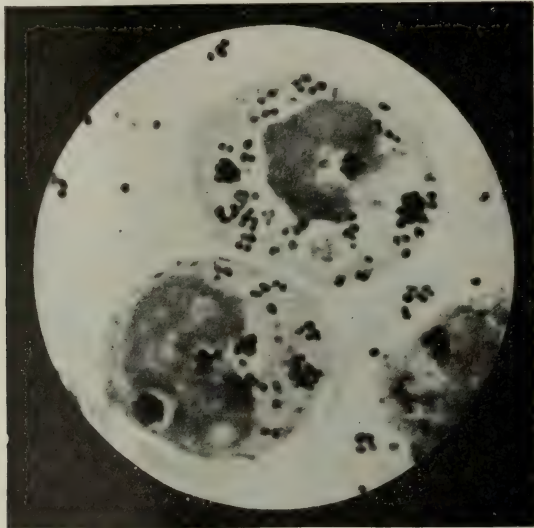
FIG. 54.—Meningococcus in leucocytes. Smear preparation from peritoneal exduate in a guinea-pig; × 2000 (Wright and Brown).

Exceptionally, we have found that the intraperitoneal injection of a bouillon suspension of a twenty-four-houl blood-serum culture in the quantity of about 1 c.c. would kirl guinea-pigs within forty-eight hours.

At autopsy there is an accumulation of a cloudy or blood-stained fluid in the peritoneal cavity, some little enlargement of the spleen, and some injection of the peritoneum. Microscopical examination of the exudate shows numerous leucocytes crowded with the diplococci (Fig. 54).

Occurrence.—Found in the meningeal exudate of epidemic cerebrospinal meningitis. It is situated mainly inside the pus-cells, some of which may contain many diplococci. In some cases the presence of the organism in the exudate may be difficult or impossible to demonstrate, and it is probable that it rapidly dies out. It has been recovered from the lungs in cases of pneumonia which sometimes accompany the disease, and in the nasal secretion both of sick and of well individuals. A general invasion of the circulation by the microorganism may sometimes be shown by cultures from the blood taken early in the course of the disease.

Diagnosis (see also section on Lumbar Puncture).—In exudates suspected of containing meningococci smear preparations should be stained by Gram's method. The presence of micrococci in the pus-cells, decolorized by Gram's method, is practically sufficient for its identification in the meningeal exudate. A few cases have been reported in which an organism has been recovered in pure culture which has possessed the culturally and serological characteristics of Micrococcus catarrhalis.

Cultures should be made on blood or laked blood agar plates. The agar should be adjusted to a reaction in the neighborhood of pH 7.6, and may or may not contain 0.5 per cent. glucose. The diagnosis can be confirmed by agglutination tests. Pure cultures in glucose "hormone" broth containing 1 per cent. calcium carbonate or suspensions of pure cultures on the various solid media are added to equal amounts of 1 : 50, 1 : 100, and 1 : 200 dilutions of a potent polyvalent antimeningococcic serum. It is desirable to control the test by using a suspension of a known meningococcus and also by using dilutions of normal serum for both organisms. The test should be read after exposure for twenty-four hours to 55° C. in a water bath. Definite agglutination in the first tube (final serum dilution 1 : 100) is considered sufficient for the diagnosis of meningococcus.

Recently Gordon[1] has divided meningococci into four types depending upon agglutination reactions and agglutinin

[1] Gordon, British Med. Research Committee Reports, London, 1915–1917.

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absorption tests.   Other workers have confirmed his results
with the qualification that many strains cannot be definitely
classified as belonging to one of the fixed types.   The organisms
have also been classified according to tropin reactions.   At the
present time all serums manufactured under the control of the
U. S. Hygienic Laboratory are made by immunizing horses with
15 strains representing the various agglutination and tropin
types.   In spite of the polyvalent character of the serum cases
occasionally occur which fail to respond to serum treatment.
In such instances, as a rule, it will be found that the thera-
peutic serum used is low in agglutinin content towards the
isolated causative organism.   If a laboratory engaged in the
manufacture of biologic products is nearby it may be possible
to secure a more potent serum either by testing various lots of
serum against the isolated meningococcus, or by determining
the type of the organism by the use of monovalent type serums
and obtaining a therapeutic serum of particularly high titer
against this type as determined by the routine potency tests
of the laboratory.

In the examination of suspected carriers material from the
nasopharynx is obtained by means of fairly long (16–20 cm.)
sterile copper wire swabs bent at the swab end.   These may
be enclosed in a piece of glass tubing bent in the same way—
the West tube.   Cultures should be made on blood or laked
blood[1] agar plates.   Transplants from suspected colonies,
provided the morphological and staining reactions are proper,
should be tested by agglutination with a potent polyvalent
serum properly controlled.   In doubtful cases sugar fermenta-
tion tests carried out in Hiss serum water media are useful for
differentiation.   Meningococci ferment dextrose and maltose
and do not ferment levulose, saccharose, lactose and galactose.
Considerable work may be saved by employing the slide-
agglutination method.   With a platinum wire portions of the
suspected colony are transferred to drops of 1:10 and 1:50

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[1] *Laked Blood Agar.*—To four parts sterile distilled water add one part sterile
defibrinated human, horse, sheep, or rabbit blood. Of the mixture add 5–10
per cent. to melted plain agar cooled to about 45° C.

dilutions of a polyvalent antimeningococcic serum and 1:10 dilution of normal serum placed on a glass slide. Microscopic agglutination in either dilution of immune serum without agglutination in the normal serum probably means meningococcus and transplants are accordingly made to confirm the diagnosis.

Bacillus of Influenza.—*Morphology.*—Very small bacilli, with rounded ends and of variable length, sometimes growing
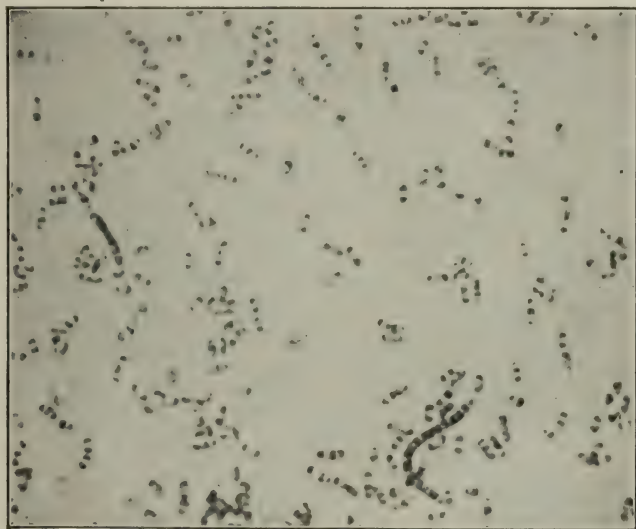
FIG. 55.—Influenza bacilli from a culture on blood-agar; × 2000 (Wright and Brown).

into long forms, more or less bent or curved (see Fig. 55).

Stains more deeply at the ends than in the middle, and in the long forms shows irregularity of staining. The faintly stained areas are very sharply defined, as in the case of the typhoid bacillus.

In smear preparations from bronchial secretions (see Fig. 56) the bacillus appears smaller and less plump than it does in preparations from cultures. It also does not show irregularities in staining.

Cultivation.—The organisms can be cultivated only in media containing blood or its derivatives. It was originally isolated

by Pfeiffer on agar slants over whose surface had been smeared several drops of blood. For this purpose human, rabbit, guinea-pig, pigeon, or frog blood was used, the best growth being obtained with pigeon blood. Good growth can be obtained on blood agar plates prepared in the usual way.

Probably the best medium for the isolation of the bacilli is sodium oleate blood agar. The sodium oleate apparently checks the growth of the various contaminating organisms and at the same time favors the growth of the influenza bacillus.
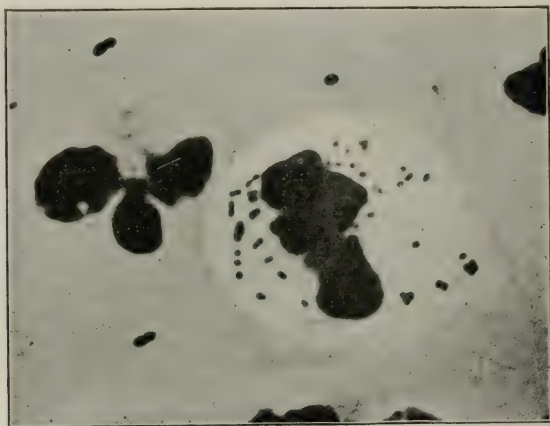
FIG. 56.—Bacilli of influenza in a leucocyte in a smear preparation from sputum. A pneumococcus also in the same leucocyte and other pneumococci free. The small size of the bacillus of influenza will be apparent by comparison with the pneumococci; × 2000 (Wright and Brown).

For continued cultivation after isolation chocolate agar or broth serve the purpose admirably.

Colonies.—After twenty-four hours in the incubator the colonies on blood agar appear as minute colorless, glassy, transparent points resembling small drops of dew. They never attain any size, and do not become confluent. They are barely visible to the unpractised eye, and require a low magnifying power to be seen clearly. Under the low magnifying power they are translucent, homogeneous, not granular, and circular in outline (Fig. 57).

Decolorized by Gram's method. Not motile. Will not grow without oxygen.

Pathogenesis.—Some strains, especially those derived from cases of meningitis, are pathogenic for white mice and rabbits.

Occurrence.—Found in the exudate of the respiratory tract in influenza, frequently inside of leucocytes (Fig. 56). It may be present in the small bronchi and in the exudate of broncho-pneumonia in this disease. It has been observed in purulent meningitis secondary to influenza. It is frequently found in mouths of normal individuals and of those suffering from disease other than influenza.

FIG. 57.—Bacillus of influenza; colonies on blood agar (F. T. Lord; photo. by L. S. Brown).

F. T. Lord, working in the Laboratory of the Massachusetts General Hospital, found influenza bacilli in 60 of 100 unselected specimens of sputa repeatedly negative for tubercle bacilli. In 29 of these 60 cases the bacilli were present in great numbers. Eleven cases were of acute and 18 of chronic inflammation of the respiratory tract. In the chronic cases he demonstrated the persistence of influenza bacilli in the sputum for months or years.

Lord believes that influenza bacilli are very commonly present in sputa apart from epidemics of influenza, and that chronic infection with influenza bacilli is not infrequently mistaken clinically for tuberculosis.

Diagnosis.—Microscopical examination of smear prepara-
tions of the bronchial sputum shows very small, short, round-
ended bacilli, often in very large numbers and frequently in the
pus-cells. These bacilli frequently occur in pairs and resemble
pairs of cocci. Their ends may be more deeply stained than
the central portions. For the staining of smear preparations of
the sputum Pfeiffer recommends that a very dilute carbol-
fuchsin solution be applied for five to ten minutes. The
smear preparation is to be made from a distinctly purulent
portion of the sputum. Staining with Löffler's methylene-blue
solution also gives good results. See also W. H. Smith's
method for staining the capsule of the pneumococcus, page 282.

The bacillus of influenza may be cultivated from the sputum
by breaking up a small portion of a distinctly purulent character
in 1 or 2 c.c. of bouillon, and then spreading a platinum loopful
of the suspension over the surface of a suitable medium, which
is then placed in the incubator. After eighteen to twenty-four
hours the characteristic colonies may be visible with the aid
of a hand-lens. These should not grow in ordinary media
unless blood or hemoglobin be present, and should have the
morphology of the bacillus of influenza.

To Stain the Influenza Bacillus in Sections.—1. Harden in
alcohol.

2. Stain half an hour or more in carbol-fuchsin diluted
with 20 parts of water.

3. Wash out in a watch-glass of water to which is added
a drop of glacial acetic acid until the section appears gray-
violet in color.

4. Alcohol, xylol, balsam.

For Zenker's fixed tissues the eosin-methylene-blue stain
is recommended.

Bacillus Pertussis (Bordet=Gengou).—The bacillus of
whooping-cough was discovered by Bordet and Gengou in
1900 in the sputum of acute cases of the disease. The first
cultures were obtained by them in 1906.

Morphology.—The micro-organism is a very small non-
motile bacillus. Frequently it is so short that it resembles

a micrococcus. It is more regularly ovoid and somewhat larger than the influenza bacillus, and shows less tendency to pleomorphism and involution. It stains readily with the ordinary aniline dyes, and usually more intensely at the ends than in the middle. It is decolorized by Gram's method.

Cultivation.—It is difficult to start a growth of the whooping-cough bacillus, although the organism grows readily
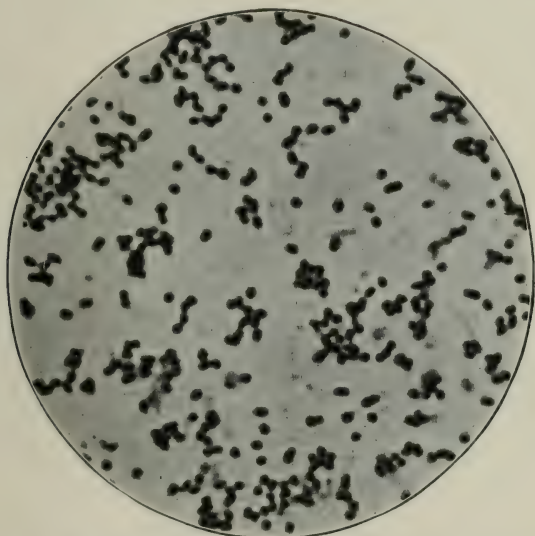
FIG. 58.—Bacillus pertussis from a culture; × 2000 (Mallory).

enough after it has become accustomed to an artificial medium. Bordet and Gengou obtained their first cultures on a special potato-blood-agar medium which they devised.

The medium is prepared as follows:

(*a*) A glycerin extract of potato is prepared by steaming in an autoclave 100 grams of sliced potato in 200 c.c. of a 4 per cent. solution of glycerin in water.

(*b*) To 50 c.c. of this extract add 150 c.c. of a 0.6 per cent. salt solution and 5 grams of agar. Melt the mixture in the autoclave, pour 2 to 3 c.c. into test-tubes, and sterilize.

(c) After sterilization add an equal volume of sterile defibrinated rabbit blood, or, preferably, human blood, mix the two fluids, slant the tubes, and solidify the mixture.

Cultures should be made from the sputum during the early days of the disease. The organisms grow slowly at first, so that the colonies are barely visible after twenty-four hours. After forty-eight hours they are plainly visible as small, grayish, rather thick colonies. Later generations grow with luxuri-
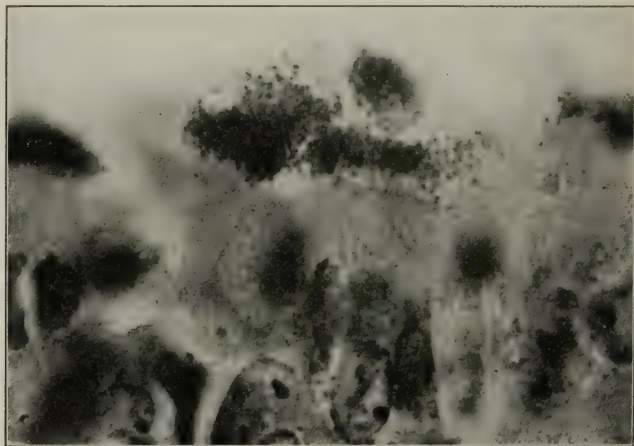
FIG. 59.—Bacillus pertussis in masses between the cilia of the epithelial cells lining the trachea; × about 2000 (Mallory).

ance. After they have been cultivated for some time on the potato-blood-agar medium they will grow readily on plain blood-agar or ascitic-agar and in ascitic broth, or broth to which blood has been added.

Occurrence.—The bacillus of whooping-cough is found in large numbers and in practically pure culture in the early stages of the infection. It occurs free in the secretion, and also within polymorphonuclear leucocytes. Study of post-mortem cases has shown that the organism is located between the cilia of the epithelial cells lining the trachea and bronchi. This location seems to be characteristic for this organism in man.

In experimental work with this bacillus on animals, especially puppies and young rabbits, it is necessary to bear in mind

that the Bacillus bronchisepticus, the cause of distemper, snuffles, etc., in animals, is often present, and that it is of the same size and occupies the same peculiar position between the cilia of the cells lining the air-passages, including the nasal cavity. It differs from the bacillus of whooping-cough in being motile and in producing alkali in litmus-milk.

Bacillus Coli Communis.—*Synonyms:* Bacterium coli commune; Colon bacillus.

Fig. 60.—Bacilli coli communis from a bouillon culture, showing the irregularity of staining of the bacillus; × 2000 (Wright and Brown).

Morphology.—A medium-sized bacillus with rounded ends, often short or even coccus-like, but may grow in long forms. Faintly staining, sharply defined areas are present in the protoplasm (Fig. 60).

Decolorized by Gram's method.

Motility.—Usually not motile, but some varieties show sluggish independent movement.

Flagella may be demonstrated by the special methods of staining. They are less numerous than in the case of the typhoid bacillus.

Blood-serum.—Rounded, grayish-white, slightly elevated, viscid-looking colonies, which may attain a diameter of 3 mm. after twenty-four hours in the incubator.

Agar.—Dirty gray to white, slightly translucent surface colonies. Usually round and moderately elevated. Sometimes irregular in outline.

Gelatin Slant.—Grayish translucent strip with wavy margins. The gelatin is not liquefied. Growth is more rapid than in the case of the typhoid bacillus.

A single colony on a gelatin plate is shown in Fig. 61.

Dextrose-gelatin Stab.—Growth along the line of stab in the form of confluent spherical colonies, and on the surface about the point of entrance of the needle as a thin gray circular layer.
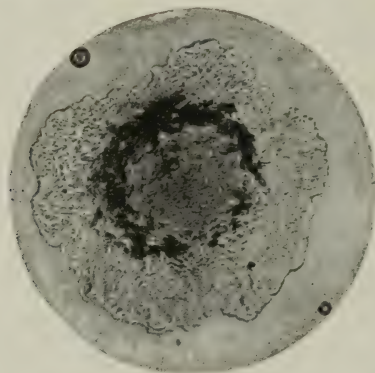
Fɪɢ. 61.—Bacillus coli communis: superficial colony two days old upon a gelatin plate; × 21 (Heim).

Gas-bubbles are produced in the gelatin from fermentation of the glucose. The gelatin is not liquefied.

Bouillon.—Markedly clouded, with formation of a sediment. The clouding is more marked than in the case of the typhoid bacillus.

Litmus-milk.—Turned pink and usually coagulated.

Action on Sugars.—Considering the more important sugars, dextrose, lactose, maltose, mannit, and dulcit are fermented with the formation of acid and gas and no acid or gas is formed with saccharose. These fermentation reactions are made use of in isolating the colon bacillus and in differentiating it from other members of the colon-typhoid-dysentery group.

Potato.—Dirty grayish or brownish, viscid-looking layer.

Dunham's Peptone Solution.—Marked indol-production. This is shown by the appearance of a red color in the culture after the addition of 5 drops of concentrated sulphuric acid, c. p., and 1 c.c. of a 1:10,000 solution of sodium nitrite. The culture in the peptone solution should have been at least twenty-four hours—or, better, forty-eight hours—in the incubator before the test is made.

Pathogenesis.—"Its virulence as tested upon animals is variable, but is generally manifest only after inoculation of large doses, which kill by intoxication rather than infection" (Welch).

The lesions produced are not sufficiently characteristic to be detailed here.

Occurrence.—Occurs constantly in the intestinal canal of human beings and animals, and is widely distributed in the external world.

"The colon bacillus is a frequent invader of the internal organs in all sorts of diseases, especially when there are intestinal lesions. It manifests no evident pathogenic action in most of these cases, and is then without clinical significance. It occurs frequently associated with other bacteria in infected wounds and other inflammations of exposed surfaces. Here also it does not usually appear to cause serious disturbance. The fact that the colon bacillus is so common and widely distributed, and found so often as a harmless invader, should lead to much caution in interpreting the significance of its presence when it occurs in definite lesions. There is no doubt, however, that it may be pathogenic for man. It plays an important rôle in inflammations of the urinary tract and biliary passages; also, but usually with less independence, in peritonitis and appendicitis.

"The list of diseases in which it may be found is a very long one, and includes inflammations in all organs and parts of the body. In general its pathogenic properties are of a mild character. One of its leading rôles is to invade territory already occupied by other bacteria or previously damaged. It may be concerned in the production of gall-stones, in the interior of

which it has been found by the writer with great frequency"
(Welch, Dennis's *System of Surgery*, vol. i.).

Diagnosis.—The bacillus above described is to be regarded as
a type of a group of bacilli constituting the so-called "colon
group" of bacilli.   From the point of view of public health the
differentiation of these organisms is often of the greatest import-
ance.   This applies particularly to the bacteriological examina-
tion of water.   According to the recommendations of the
American Public Health Association[1] this group includes all
Gram decolorizing short bacilli which do not form spores or
liquefy gelatin and which are facultative anaerobes and ferment
dextrose and lactose with the formation of acid and gas.
Bacillus coli communior, Bacillus lactis aërogenes, and Bacillus
acidi lactici are placed in this group in addition to Bacillus
coli communis.   Differentiation is accomplished by means of
the two sugars—saccharose and dulcit.   The former is fer-
mented by B. coli communior and B. lactis aërogenes with the
formation of acid and gas, and the latter by B. coli communis
and B. coli communior with the formation of acid and gas.

Bacillus Typhosus.—*Synonyms:* Bacillus typhi abdomin-
alis; typhoid bacillus.

Morphology.—Medium-sized bacilli with rounded ends,
generally short (Fig. 62), but sometimes long or thread-like,
and frequently showing faintly stained, sharply defined areas
in their protoplasm (Figs. 62 and 63).

Decolorized by Gram's method.   Does not form spores.

Motility.—Very marked.

Flagella (Fig. 65) may be demonstrated by the special
methods of staining described elsewhere.

Blood-serum.—Round, grayish, viscid-looking colonies, which
may attain a diameter of 2 mm. after forty-eight hours in the
incubator.

Agar.—Round, elevated, grayish colonies.   At first slightly
transparent, later more opaque.

Bouillon.—Clouded, with the formation of some sediment.
The clouding of the medium is not so marked as in the case

[1] A. P. H. A., *Standard Methods of Water Analysis*, 1915.

of Bacillus coli communis. In general, the growth of the typhoid organism is not so vigorous on culture-media as is the growth of Bacillus coli communis.

FIG. 62.—Typhoid bacilli from a bouillon culture, showing characteristic irregularity in staining and variability in length; × 2000 (Wright and Brown).

Gelatin Slant.—Broad translucent streak with wavy, irregular margins. The gelatin is not liquefied. Growth is slower than that of Bacillus coli communis in the same medium.
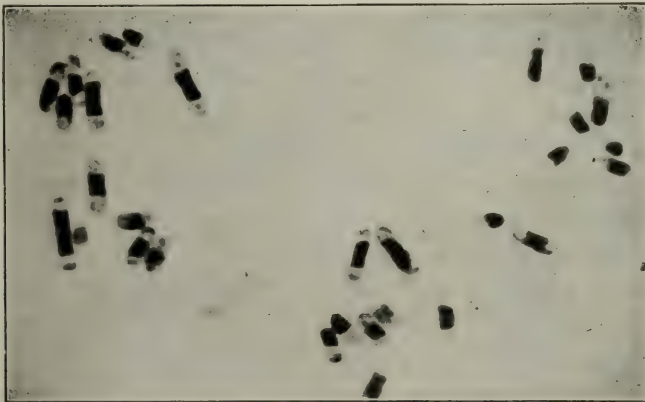
FIG. 63.—Typhoid bacilli from a culture on potato, showing unstained areas in the bacilli and polar granules; × 2000 (Wright and Brown).

An isolated colony, slightly magnified, on gelatin, is shown in Fig. 64.
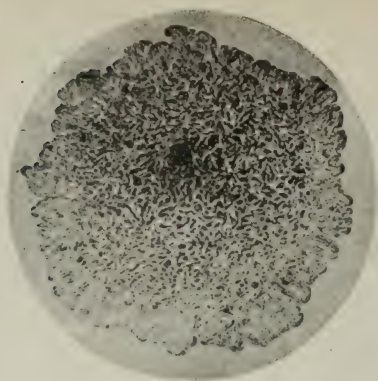
FIG. 64.—Bacillus of typhoid fever: superficial colony two days old, as seen upon the surface of a gelatin plate; × 20 (Heim).
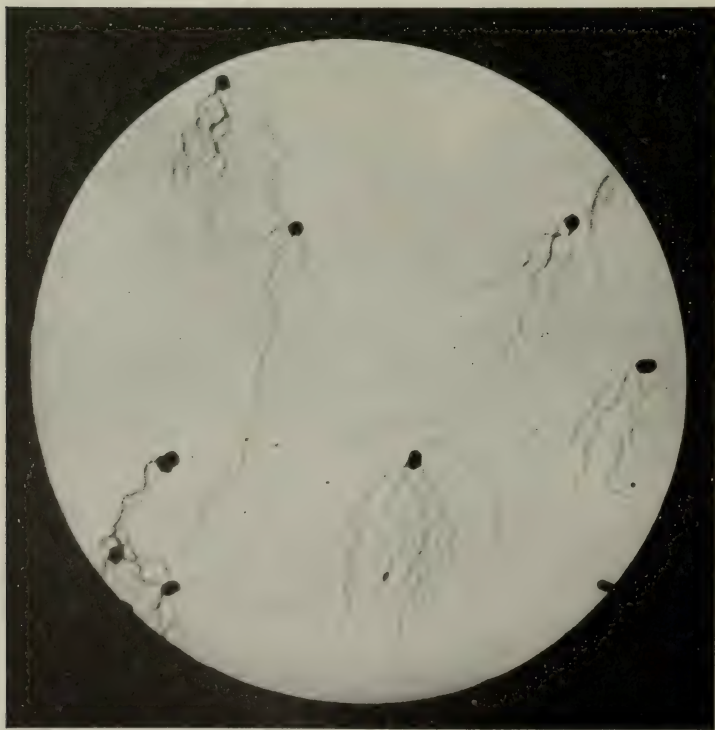
FIG. 65.—Typhoid bacilli, from a culture on agar, showing flagella, from a preparation stained by Dr. Hugh Williams; × 2000 (Wright and Brown).

Dextrose-gelatin Stab.—Growth all along the line of inoculation in the form of confluent spherical grayish colonies, and on the surface about the point of entrance of the platinum wire in the form of a circular translucent grayish layer. No production of gas-bubbles. No liquefaction.

Litmus-milk.—No visible change.

Action on Sugars.—In media containing glucose, maltose, or mannite acid is produced, but no gas. Neither is produced in media containing lactose, rhamnose, or saccharose. In media containing dulcit there is a slow production of acid without gas.

Potato.—Growth occurs, but it is usually invisible.

Dunham's Peptone Solution.—No indol-production—*i.e.* no red color appearing in twenty-four to forty-eight-hour cultures after the addition of 5 drops of concentrated sulphuric acid, c. p., and 1 c.c. of a solution of sodium nitrite, 1 : 10,000.

Pathogenesis.—The inoculation of animals is usually without results if moderate quantities of the organism are used. Sometimes, however, death occurs apparently from the effects of the toxic material injected.

Occurrence.—Found in the spleen in large numbers at autopsies in typhoid fever. Its presence may also be demonstrated in the intestinal lesions, rose spots, mesenteric lymph-nodes, liver, bile, kidneys, urine, and blood. As a rule, the number of bacilli found in the liver, kidneys, and blood is small. In the bile they may be numerous and may persist in it for a long period of time after the disease has subsided. In some cases the urine contains enormous numbers of the bacilli.

The typhoid bacillus may also occur in the suppurative sequelæ of typhoid fever, especially those involving bones. In these conditions, however, it may be accompanied by the pyogenic cocci. During the disease the organisms are frequently recovered from the blood. The percentage of positive findings diminishes as the duration of the disease increases. Coleman and Buxton were able to obtain positive blood cultures in 89 per cent. of their cases during the first week, whereas after the fourth week typhoid bacilli were recovered in

only 26 per cent. of the cases. The organisms appear in the feces as a rule rather late in the course of the disease, but may persist for a long time after recovery.

Typhoid bacilli may be recovered from contaminated water supplies and from cow's milk which has been contaminated either from handling by a typhoid carrier or from being placed in bottles returned from houses where typhoid fever or carriers exist. Their presence has been reported in oysters contaminated by sewage.

To Stain for B. Typhosus in Sections.—Typhoid bacilli in stained sections are generally best hunted for with a low power. The characteristic colonies which they form are easily recognized. Good results in staining can be obtained with Löffler's methylene-blue solution used in the manner already described, but the stain is never very intense. For rendering the bacilli rather more prominent, so that small groups of them may be recognized, Flexner has advised the two following methods:

A.—1. Stain paraffin sections in Löffler's methylene-blue solution for two hours.

2. Acetic-acid solution, 1 : 1000, for several minutes.

3. Dehydrate in absolute alcohol.

4. Oil of cloves to clear and differentiate.

5. Xylol, several changes.

6. Xylol balsam.

B.—1. Stain sections in Sterling's gentian-violet solution for ten minutes.

2. Acetic-acid solution, 1 : 1000, for some minutes.

3. Dehydrate quickly in 95 per cent. alcohol.

4. Transfer to slide, blot, add oil of cloves to clear, and differentiate. Change the oil several times until the desired differentiation is obtained.

5. Wash off section several times with xylol.

6. Xylol balsam.

Cultivation of the Typhoid Bacillus from the Blood during Life.—Blood cultures are made in the routine way by adding about 5 c.c. of blood to 50–100 c.c. of bouillon. If Gram decolorized bacilli are found after incubation the subsequent

steps are identical with those following the isolation of suspicious colonies from feces and other grossly contaminated material (see page 340).

The Ox-bile Method.—Test-tubes containing 5 c.c. of plain ox-bile sterilized by steam are used. If a precipitate appears on heating, it may be filtered off and resterilized before use without detriment to the medium. The blood of the patient is added to the bile in the test-tube up to 2.5 c.c. in amount. The mixture of bile and blood is then incubated at 37° C. for twelve to fifteen hours, when transfers of a few loopfuls are made to other culture-media in order to establish the identity of the bacteria that may have developed.

Diagnosis.—See page 340.

Bacilli Paratyphosi A and B and Related Bacilli.— There are a certain group of intermediary organisms between the colon group and B. typhosus. Morphologically and culturally they may resemble one or the other. In this group are included B. paratyphosus A, B. paratyphosus B, B. enteritidis, B. abortus, B. typhi murium, and the bacillus of hog cholera. The first two, rarely the third, are important from the point of view of the clinical bacteriologist for these bacilli have been found associated with inflammatory processes and with fevers resembling typhoid fever. The B. paratyphosus A strains group themselves serologically into a rather definite group, whereas the so-called B. paratyphosus B group contains many variants. In general the paratyphoid strains grow more luxuriantly than the typhoid bacillus and produce acid and gas in media containing dextrose or mannit. They produce acid in rhamnose without gas production and xylose is fermented without gas by B. paratyphosus B but not by B. paratyphosus A. The most accurate differentiation between this group and the typhoid bacillus is by means of specific agglutinating immune serums (see page 341).

Bacillus Dysenteriæ.—Since the discovery of the dysentery bacillus by Shiga in 1898 many strains have been recovered which do not agree either with Shiga's bacillus or with each other either in sugar fermentations or serological reactions.

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Hiss[1] has divided them into four groups according to their ability to ferment various sugars. Group I ("Shiga," "Kruse," and "New Haven") ferments dextrose. Group II ("Y" (Hiss and Russell) "Seal Harbor," "Diamond," and "Ferra") ferments dextrose and mannit. Group III ("Strong") ferments dextrose, mannit, and saccharose, and Group IV ("Harris," "Gray," "Baltimore," and "Wollenstein") ferments dextrose, mannit, maltose, saccharose (usually only after six days), and dextrin. Hiss also found that the serological reactions of the various strains ran true to this grouping, that is, an immune serum of any strain in a group agglutinated all other strains in the same group in high dilution, whereas all strains in the other groups were agglutinated only in relatively low dilutions. All organisms with the general morphological and cultural characteristics of dysentery bacilli and recovered from cases of clinical "dysentery" will not fall definitely into the above groups. In any such group of closely related organisms there are always many variants.

Morphology.—This bacillus resembles the typhoid bacillus in morphology, but in general it is plumper and less frequently appears in filamentous forms. Involution forms quickly develop in glucose-agar cultures. It is decolorized by Gram's method, and does not form spores. It is not motile. The bacillus grows in bouillon and on agar and gelatin, both in plate and tube cultures, with appearances very similar to those of the typhoid bacillus.

Pathogenesis.—The bacillus is pathogenic for the usual laboratory animals, especially for mice and guinea-pigs, which may die in from twenty-four to forty-eight hours after subcutaneous or intraperitoneal inoculation. At autopsy there may be found local inflammation at the seat of inoculation, ecchymoses of the serous membranes, serous or serohemorrhagic exudate in the pleural or peritoneal cavities, enlargement of the spleen, and hyperemia or hemorrhage in the intestinal walls.

Occurrence.—In the stools, intestinal contents, and in the ulcerated mucous membrane of acute dysentery, whether

[1] Hiss, *The Journal of Medical Research*, 1904, viii, 1.

sporadic or epidemic. It may be found in the mesenteric lymphatic glands, but is not found in the blood, in the spleen, or in other viscera.

Isolation and Differentiation of Colon, Typhoid, Paratyphoid, Dysentery, and Closely Related Organ= isms.—The differentiation of these various Gram decolorized bacilli isolated most commonly from fecal material depends in general upon the consideration of their motility and their action on media containing different sugars. Definite identification is made by the use of specific immune serums. The accompanying table has been copied from the text book of Hiss-Zinsser and Russell.[1] It gives in composite form the characteristics employed in the differentiation of the organisms of these groups.

COMPOSITE TABLE OF MOST IMPORTANT TYPES[1]

	Dextrose	Mannit	Maltose	Lactose	Xylose	Rhamnose	Saccharose	Dulcit	Indol	Motility
B. coli com'is	⊕	⊕	⊕	⊕	×	×	−	⊕	+	+
B. coli com'ior	⊕	⊕	⊕	⊕	×	×	⊕	⊕	....	+
B. acidi lactici	⊕	⊕	⊕	⊕	×	×	−	−	....	±
B. lactis aerog	⊕	⊕	⊕	⊕	×	×	⊕	−	....	±
B. parat. "A"	⊕	⊕	⊕	−	−	+	−	slow	Generally no. Indol though literature not consistent on this point.	+
B. parat. "B"	⊕	⊕	..	−	+	+	−	+		+
B. enteritidis	⊕	⊕	..	−	+	+	−	+		+
B. abortus	⊕	⊕	..	−	+	+	−	+		+
B. hog cholera	⊕	⊕	..	−	+	+	−	irreg.		+
B. typhi murium	⊕	⊕	..	−	+	+	−	+		+
B. typhosus	+	+	+	−	slow	−	−	slow	−	+
B. dys. Shiga	+	−	−	−	×	×	−	−	−	−
B. dys. Flexner	+	+	+	−	×	×	−	−	+	−
B. dys. "Y" (Hiss-Park)	+	+	−	−	×	×	−	×	+	−
B. dys. Strong	+	+	−	−	×	×	+	×	+	−
B. faec. alkal	−	×	×	×	×	×	×	×	..	+
B. Morgan, No. 1	⊕+ slight gas	−	−	−	?	?	−	−	+	+

⊕ = acid and gas   − = negative
+ = acid, no gas   × = not needed for identification
[1] Copied with slight modification from "A Textbook of Bacteriology" by Hiss-Zinsser and Russell, Fifth Edition, by kind permission of the Publisher, D. Appleton and Company

[1] Hiss-Zinsser and Russell, "A Textbook of Bacteriology," D. Appleton and Co., 1922.

Given an isolated colony of a Gram decolorized bacillus, the identification is relatively a simple matter. However, the isolation of typhoid, paratyphoid, or dysentery bacilli from material grossly contaminated, such as feces, with all sorts of other organisms, including the colon bacillus, offers many difficulties.

Isolation from Feces and Other Grossly Contaminated Material. The examination should be made immediately; typhoid bacilli disappear in more than 10 per cent. of the specimens of feces in twenty-four hours. For isolation cultures are made on media which is favorable to the growth of the organisms desired and unfavorable to other organisms (Krumwiede brilliant green agar medium) or which because of its ingredients offers a gross means of differentiating between the various groups of Gram decolorized bacilli (Endo medium). Make a suspension of feces in broth or salt solution to about the viscosity of thin cream and allow the coarser particles ten to twenty minutes to settle. Place a loopful of this dilution on two brilliant green agar plates or one Endo plate or on all three. For the isolation of dysentery bacilli Endo medium alone should be used. With a sterile platinum loop or bent glass rod spread the material over the surface of the plate and continue to at least one more plate of each kind of media using the same spreader without burning it off. For purposes of comparison control plates of the media with known strains of the bacilli in pure culture should always be made. After eighteen to twenty-four hours incubation characteristic colonies should be picked and positively identified. Preliminary slide agglutination tests in a 1:100 dilution of a high titer antityphoid or other immune serum is recommended.

In examining urine the undiluted specimen or sediment after centrifuging should be plated as described above.

Identification.—Given a single colony which culturally and morphologically is consistent with the group of organisms under consideration transplants are most commonly made to Russell double sugar agar. The tubes are inoculated by smearing the surface of the slant and by making a stab into the butt. After

incubation the colon group shows acid on the slant and acid and gas in the butt, the paratyphoid bacilli show acid and gas in the butt with the slant unchanged, and typhoid and dysentery bacilli show only acid in the butt.

Another very satisfactory and simple method is to transfer the suspected colony to Dunham's pepton water medium or sugar free broth containing Andrade indicator and 1 per cent. of one of the various sugars. The media should be put up either in Smith fermentation tubes or in ordinary test-tubes containing an inverted small vial or test-tube about 5 cm. in length. With the latter the presence of any gas in the closed tube at the time of incubation should be noted. Three tubes—dextrose, lactose, and saccharose—are sufficient to differentiate the main groups. The colon group produces acid and gas in dextrose and lactose and often in saccharose. The paratyphoids produce acid and gas in dextrose, and typhoid and dysentery bacilli produce acid without gas in dextrose. Motility tests can be made to differentiate the dysentery group from typhoid bacilli. Similar media containing mannit, maltose, xylose, rhamnose, and dulcit may be used to further subdivide the larger groups.

Final identification should be made by agglutination reactions using the proper antibacterial serum as indicated by the preliminary tests. Every laboratory should keep on hand high titer antityphoid and antiparatyphoid A serums. This applies also to the group antidysentery serums in localities where frequent examinations for dysentery bacilli are likely. The antibacterial serums can be readily produced in rabbits by the subcutaneous or intravenous injection of the proper bacterial suspensions. The agglutinating strength of each serum both against its specific organism and against those of the closely related groups should be determined, and a definite diagnosis should not be made unless specific agglutination occurs in a dilution at least twice as great as the highest dilution in which group agglutination has been observed. In the absence of serum of known strength control series with pure cultures of known organisms must be run simultaneously with the test.

In actually carrying out the tests 0.5 to 1.0 c.c. of young bouillon cultures or of suspensions of the organisms in sterile salt solution are added to equal amounts of the serum dilutions contained in clean, preferably sterile, agglutination tubes. It should be borne in mind that the final serum dilution is always twice as great as the original dilution. A mixture of organisms and an equal amount of sterile salt solution should always be made as a control. The serum dilutions should be made in sterile salt solution. All tubes are incubated one or two hours in a water bath at 37.5° C. and are read immediately or after being allowed to stand overnight in an ice-box.

Bacillus Proteus (Proteus Vulgaris).—*Morphology.*— Bacilli of very variable length, sometimes appearing like cocci or as filaments.

Motile, being provided with terminal flagella. Does not stain by Gram's method.

Colonies in Gelatin.—Rapid growth with liquefaction of the gelatin. In a medium containing 5 per cent., instead of 10 per cent., of gelatin prolongations from the margins of the colonies may be formed. These may become separated from the mother colonies and form daughter colonies.

Gelatin Stab.—Rapid liquefaction along the line of inoculation with cloudiness of the liquefied gelatin and a flocculent deposit.

Agar Slant.—Widely spreading, thin, moist, grayish-white layer.

Potato.—Dirty white, moist layer.

Litmus-milk.—Turned pink and slowly coagulated.

Odor.—The cultures generally have a putrefactive odor.

Pathogenesis.—Intravenous, intraperitoneal, or intramuscular inoculations of rabbits may produce death in twenty-four to thirty-six hours after moderately large doses. Liquefied gelatin-cultures are said to be more virulent than bouillon cultures. Guinea-pigs seem to be less susceptible than rabbits to infection with this organism.

Occurrence.—This bacillus and its varieties are among the most common and widely distributed putrefactive bacteria.

It occurs in the intestinal contents. In pathological examinations it may be found in peritonitis and in abscesses, usually associated with other bacteria. It may rarely invade the circulating blood.

The so-called "proteus group" includes several varieties of similar organisms—viz., the *Proteus vulgaris*, the *Proteus mirabilis*, and the *Proteus Zenkeri*. The latter does not liquefy the gelatin, while the *Proteus mirabilis* liquefies it slowly.

Bacillus Diphtheriæ.—*Morphology.*—Bacilli varying markedly in size and shape, of irregular outline, and showing great
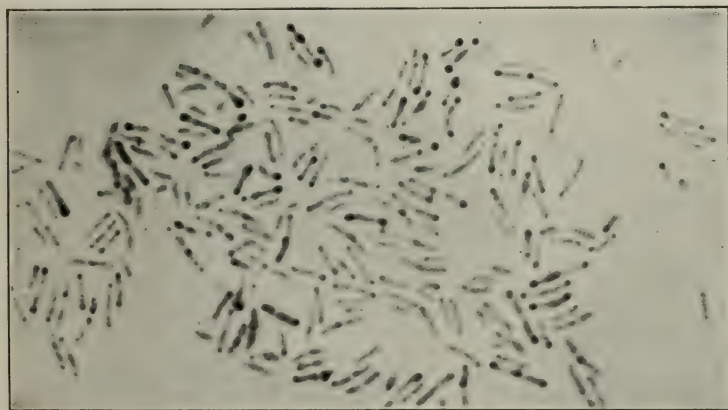
Fig. 66.—Diphtheria bacilli from a culture on blood-serum, stained by Löffler's methylene-blue solution, showing deeply stained points; × 2000 (Wright and Brown).

variability in the staining of different parts of their protoplasm (Figs. 66, 67). The presence in a palely tinted rod of deeply stained granules and points, frequently situated at the extremities, and the occurrence of irregular forms, often club-like in shape, with a constriction in the middle, are appearances which are very characteristic of the bacillus when grown upon blood-serum and stained with Löffler's methylene-blue solution. Its morphology and staining peculiarities are so characteristic when cultivated upon blood-serum that the microscopical

examination is in most cases sufficient for its identification. When grown upon other culture-media than blood-serum, however, its morphology and staining peculiarities are not so characteristic, and they may vary markedly in different media.

Stained by Gram's method.　Not motile.
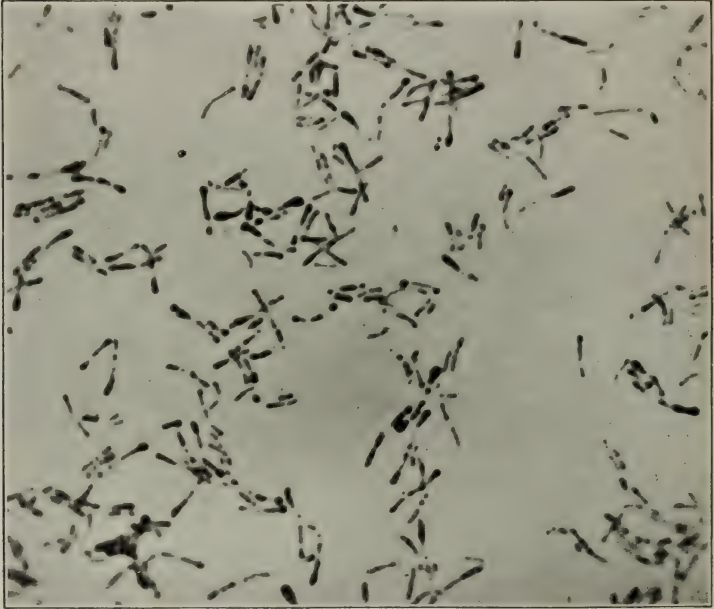
FIG. 67.—Diphtheria bacilli from a culture on blood-serum, stained by Löffler's methylene-blue solution, showing long and irregularly shaped forms of the bacillus, as well as the irregularity of staining; × 2000 (Wright and Brown).

Blood-serum.—Round, elevated, smooth colonies of the color of the medium. They may attain a diameter of 2 mm. after forty-eight hours in the incubator (Fig. 68).

Bouillon.—Growth as a rule most luxuriant at the surface with pellicle formation. In old cultures the pellicle breaks and forms fragments which settle down to the bottom of the tube. The reaction of the medium rapidly becomes acid, but changes to alkaline after a variable length of time. The presence of 1 per cent. dextrose favors rapid growth, but the organisms die out quickly presumably because of acid production.

Potato.—Growth not visible to the naked eye. The bacillus grows, however, to a certain extent, and usually assumes very atypical and irregular forms (involution forms).

Agar and Gelatin.—The growth on these media is slower and more feeble than upon blood-serum. It presents nothing remarkable.

Action on Sugars.—Produces acid in media containing dextrose or dextrin. No acid formed in the presence of saccharose.

Pathogenesis.— Subcutaneous inoculations of guinea-pigs are fatal in thirty-six to seventy-two hours in the case of virulent cultures. The lesions produced consist usually of edema, hemorrhage, and fibrino-purulent exudation about the point of inoculation in the subcutaneous tissue, hemorrhagic enlargement of the lymphatic glands, congestion and edema of the lungs, hemorrhages into the suprarenal glands, and less frequently necrosis of the liver and pleural effusions. Histological examination of the lymph-glands shows marked "fragmentation" of the nuclei of the cells, giving rise to numerous deeply staining globules of chromatin scattered throughout them. The bacilli are ordinarily found only about the point of inoculation, but cultures from the various organs will sometimes show the presence of the bacilli in some of them. Dogs, cats, rabbits, fowl, and horses are susceptible. Rats and mice are resistant.

FIG. 68.— Bacillus diphtheriæ; agar culture (photo. by Dr. Henry Koplik).

Toxin-production.—The effects produced by infection with the Bacillus diphtheriæ are due to the action of a so-called toxalbumin or toxin which the organism manufactures in its growth. The poisonous substance is produced in cultures. Its presence may be demonstrated by inoculating an animal with a small quantity of the filtrate, obtained by passing a bouillon culture some days old through an unglazed porcelain filtering apparatus, by which all the bacteria are removed from the fluid.

The toxin is contained in solution in the filtrate. If this be fairly rich in toxin, the injection of $\frac{1}{10}$ c.c. subcutaneously into a guinea-pig should lead to the death of the animal in three or four days with the various lesions above described. The local reaction, however, is not so marked as in the case of inoculation with the bacilli. With ordinary bouillon the production of a great amount of toxin by the growth of the diphtheria bacilli in it is very uncertain. Theobald Smith has shown that this uncertainty is due to the presence of variable amounts of muscle-sugar from the meat used in the preparation of the bouillon, and that this substance prevents the accumulation of toxin. He has found that that bouillon yields the most toxin which has the least muscle-sugar in it. He prepares such bouillon as follows: "Beef infusion, prepared either by extracting in the cold or at 60° C., is inoculated in the evening with a rich fluid culture of some acid-producing bacterium (B. coli) and placed in the thermostat. Early next morning the infusion, covered with a thin layer of broth, is boiled, filtered, pepton and salt added, and the neutralization and sterilization carried on as usual." This bouillon is placed in two 500 c.c. Erlenmeyer flasks, 250 c.c. in each flask. In these, cultures are made and kept for at least six or eight days in the incubator. After this time a fair amount of toxin may be assumed to have developed, and the contents of the flask are then filtered through a porcelain cylinder. The filtrate containing the "toxin" can be preserved by the addition of 0.5 per cent. pure carbolic acid.

Occurrence.—The diphtheria bacillus occurs in the local lesions in all cases of true diphtheria, in rhinitis fibrinosa and in otitis media. It may persist in the mucous membrane of the throat and nose long after convalescence has been established. The organism may be recovered from the mouths of normal healthy individuals in about 1 or 2 per cent. of all examined. Of this number about 10 per cent. usually prove to be virulent.

In fatal cases of diphtheria the organism is nearly always present in the lungs, and it may be rarely found by culture-methods more or less generally distributed in comparatively small numbers throughout the internal organs. In the majority of diphtheria autopsies an invasion of the blood-stream by streptococci, and sometimes by other bacteria, may be demonstrated by cultures. The bacillus may also be found in company with other bacteria in ulcerated or excoriated surfaces on the skin, as well as in other suppurative processes, in

individuals affected with diphtheria, and on the soiled linen of the patient. The infection of wounds with the Bacillus diphtheriæ has also been observed without coincident diphtheria.

Diagnosis.—The bacteriological diagnosis of infection with Bacillus diphtheriæ depends upon the characteristic morphology and peculiarities of staining, as well as rapidity of growth, which this organism presents when cultivated upon coagulated blood-serum. The identification by direct smear examination of the exudate is very uncertain.

The method is as follows: A blood-serum culture-tube is inoculated with a small amount of the material from the mucous membrane affected, and is placed in the incubator twelve to eighteen hours. After this length of time the resulting growth is examined by smear preparations stained either with Löffler's methylene-blue solution or by one of the special methods given below.

The diphtheria bacillus, if present, may then be recognized and differentiated from other bacteria present in the preparation by its characteristic morphology and peculiarity of staining, described on page 349. The gross appearances of the culture present little that is characteristic, as a rule, and the main reliance is to be placed on the microscopic examination. Early in the infection the greater part of the growth may be made up of the specific bacilli, but toward convalescence they fall into the minority. The ordinary forms of agar culture are not suitable for use in the bacteriological diagnosis of diphtheria, owing to the comparative feebleness of the growth of the organism on these media, and because of the fact that its microscopic appearances when cultivated on such media are not sufficiently characteristic.

The material for culture is very conveniently obtained by means of sterilized cotton swabs. In collecting this material the swab is removed from its test-tube and touched to the affected areas of the mucous membrane of either the nose or throat. It is then to be gently rubbed over the surface of a blood-serum culture-tube, or it may be replaced in the test-tube and the inoculation of the culture-tube made later in

the laboratory. In the latter case the inoculation should be made within an hour or two after the material has been collected, the infected swab meanwhile being prevented from drying by firmly replacing the cotton plug.

In cases with membrane-formation the greatest number of bacilli are on the surface or in the upper layer of the membrane, and the swab should therefore be touched to these portions rather than to the tissue beneath.

Virulence Tests.—For the isolation of the suspected organism in pure culture blood agar plates should be used. The swabs are smeared directly over the surface of the plates or are immersed in a tube of bouillon, plating a loopful of the dilution. After incubation over night suspicious colonies are picked. The desired colonies are elevated, discreet, translucent, and grayish with slightly irregular margins. There is no hemolysis. Smear preparations should be stained both with Löffler's methylene-blue solution or one of the special granule stains and with the Gram stain. The latter serves to differentiate the irregular staining Gram decolorized bacilli which are frequently found in the discharges from cases of chronic otitis media. Transplants should be made to blood-serum and tested the following day for purity.

Providing a pure culture has been obtained transplants are made to plain bouillon or veal broth, preferably sugar free, and also to sugar free broth, Hiss serum water, or pepton water containing 1 per cent. dextrose. If no acid is produced in the latter after seventy-two hours' growth, the organism is not Bacillus diphtheriæ. Maximum toxin production occurs in sugar-free plain bouillon or veal broth adjusted to pH 7.4 to 7.8 and contained in Erlenmeyer flasks in such amounts that the media forms a thin layer, thus affording free access of oxygen.

Two cubic centimeters of a forty-eight to seventy-two hour culture in the bouillon or veal broth are injected subcutaneously into a normal 250-gram guinea pig. A similar injection is made into a control pig which should also receive 250 to 400 units of diphtheria antitoxin simultaneously. Some workers prefer to administer the antitoxin to the control pig twelve

to twenty-four hours previous to the toxin injection. If the diphtheria bacillus in question is virulent the test pig will die in thirty-six to ninety-six hours with typical toxic lesions, whereas the control pig will survive.

If many tests are to be performed the use of the intracutaneous test results in a considerable saving of guinea pigs. For this purpose the twenty-four hour growth on a slant of Löffler's blood serum is emulsified in 20 c.c. of sterile salt solution and 0.15 c.c of this suspension is injected *intracutaneously* into two normal guinea pigs at corresponding points on the abdominal walls, which have previously been closely shaved or depilated by other means. One of the pigs should have received 250 to 400 units of diphtheria antitoxin intraperitoneally twenty-four hours previous to the toxin injection. The antitoxin may be administrated intracardially simultaneously with the toxin injection. Six or eight separate tests can be made with each pair of pigs. With virulent organisms local erythema, and induration with subsequent superficial necrosis develops within two or three days on the test pig, whereas the skin of the control pig remains normal.

Special Methods of Staining the Bacillus Diphtheriæ.—Owing to the fact that the diphtheria bacillus may be recognized by its peculiar morphology and characteristic staining in smear preparations from its growth upon certain culture-media, as already pointed out, various special staining methods have been devised for accentuating and rendering more striking to the eye the peculiar deeply stained points and granules in the bodies of the individual bacilli, which have been referred to as of great importance in the identification of the organism.

These special methods of staining are said to be of great advantage in cases where only a few specific bacilli may be suspected to be present among a large number of other bacteria.

Neisser's Method.—1. Stain for one to three seconds in a solution which is made as follows: 1 gram of methylene-blue, in powder, is dissolved in 20 c.c. of 96 per cent. alcohol. To this add 950 c.c. of distilled water and 50 c.c. of glacial acetic acid, and filter.

2. Wash in water.

3. Stain for three to five seconds in a solution of vesuvin (Bismarck brown), made by dissolving 2 grams of the dye (in powder) in 1000 c.c. of boiling distilled water.

4. Wash in water, and mount.

The diphtheria bacilli stained by this method appear as pale brown rods bearing bluish-black granules, usually of oval shape and of a diameter somewhat greater than the rod. The majority of the bacilli show a granule at each end or at only one end, but not rarely three granules are present, one being near the middle of the rod. More granules than these are exceptional (see Fig. 69).

The bacilli must have been grown on Löffler's blood-serum medium, and the culture must be at least nine hours and not more than twenty-four hours old.
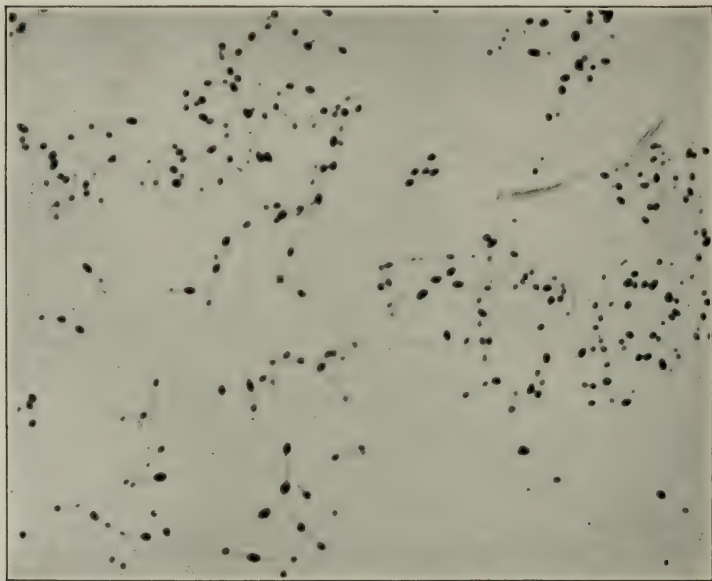
Fig. 69.—Diphtheria bacilli from blood-serum culture stained according to Neisser's method; × 2000 (Wright and Brown).

Hunt's Method.—1. Stain in saturated aqueous solution of methylene-blue one minute without heating.

2. Wash in water.

3. Cover with aqueous solution of tannic acid, 10 per cent., for ten seconds.

4. Wash in water.

5. Stain in saturated aqueous solution of methyl-orange one minute, without heating.

6. Wash in water.

7. Dry, and mount in balsam.

By this method the granules, etc., are dark blue or almost black, and stand out very sharply against the light-green coloring of the body of the bacillus (see Fig. 70). The solution of methyl-orange should be freshly prepared, for it deteriorates in a few days.

Mallory's Stain for the Diphtheria Bacillus.—This staining method was devised because Löffler's solution made up with some of the methylene-blues now in the market stains
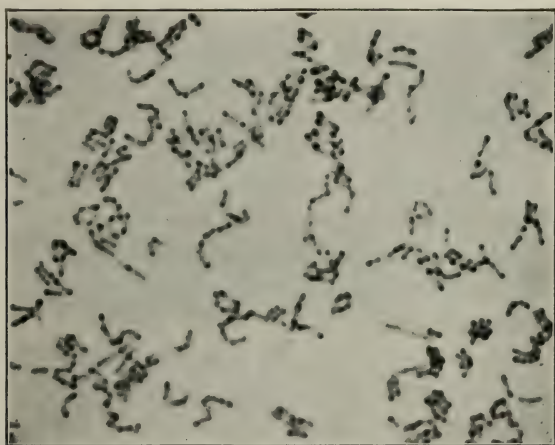
FIG. 70.—Diphtheria bacilli from blood-serum culture stained according to Hunt's method; × 2000 (Wright and Brown).

poorly and does not keep well. Extended use of this solution shows that it keeps perfectly and can be used repeatedly.

Methylene-blue,	1 gm.;
Water,	100 c.c.;
Glacial acetic acid,	3 "

Stain smear preparations 15 to 30 minutes; wash off with water and dry. Overstaining does not occur. The polar bodies or granules are stained intensely. The body of the bacterium stains lightly. The solution cannot be recommended as a general stain for bacteria because it is not intense enough, unless structures similar to polar bodies are present.

Pseudodiphtheria and Diphtheroid Bacilli.—These two groups of organisms are important chiefly because of their morphological resemblance to the diphtheria bacillus. The

former consist of two definite organisms, B. Hoffmanni and B. xerosis, while the latter consist of a large heterogenous unclassified group.

B. Hoffmanni may be recovered from the throats of normal individuals.   Morphologically it is shorter and fatter than the diphtheria bacillus and granules cannot be found when stained with the special stains.   It grows readily on the simplest of culture media, and may be readily distinguished from the diphtheria bacillus in that it does not produce acid in media containing dextrose or dextrin.   It is non-pathogenic.

B. xerosis is recovered frequently from the conjunctivæ of normal and slightly inflamed eyes.   Morphologically and culturally it closely resembles the diphtheria bacillus.   Aside from not producing, toxin, it may be differentiated from the diphtheria bacillus from the facts that it produces acid in media containing saccharose and does not produce acid in media containing dextrin.

Diphtheroid bacilli which morphologically and culturally resemble the diphtheria bacillus can be differentiated only by their failure to produce toxin.

Scarlet Fever.—Although many workers have isolated various organisms from cases of scarlet fever, it must be acknowledged that the etiology is unknown.   Undoubtedly streptococci are in some way closely related to the disease. Streptococci are very frequently found in the blood stream in those cases which are acutely fatal, and the work of Moser and Von Pirquet[1] and more recently of Bliss[2] and of Tunnicliff[3] tends to show that streptococci isolated from scarlet fever cases fall into a rather specific serological group and that there are present in the serum of convalescents specific antibodies to this homologous group.

Mallory and Medlar[4] have isolated a diphtheroid bacillus from the upper respiratory tract, bronchi, and alveoli.   They

[1] Moser and von Pirquet, *Cent. f. Bakt., Orig.*, 1903, xxxiv, 714.

[2] Bliss, *Bull. Johns Hopkins Hospital*, 1920, xxxi, 173.

[3] Tunnicliff, *J. Inf. Dis.*, 1921, xxix, 91; and *J. A. M. A.*, 1920, lxxiv, 1386 and lxxv, 1339.

[4] Mallory and Medlar, *J. Med. Research*, 1916, xxxv, 209.

were able to demonstrate clumps of the organisms lying superficial to necrotic epithelial cells of the upper respiratory tract (Fig. 71). More recently Parker[1] has recovered a quite similar organism consistently from discharging ears complicating the disease. Filtrates of bouillon cultures of the latter contain a moderately powerful poison.
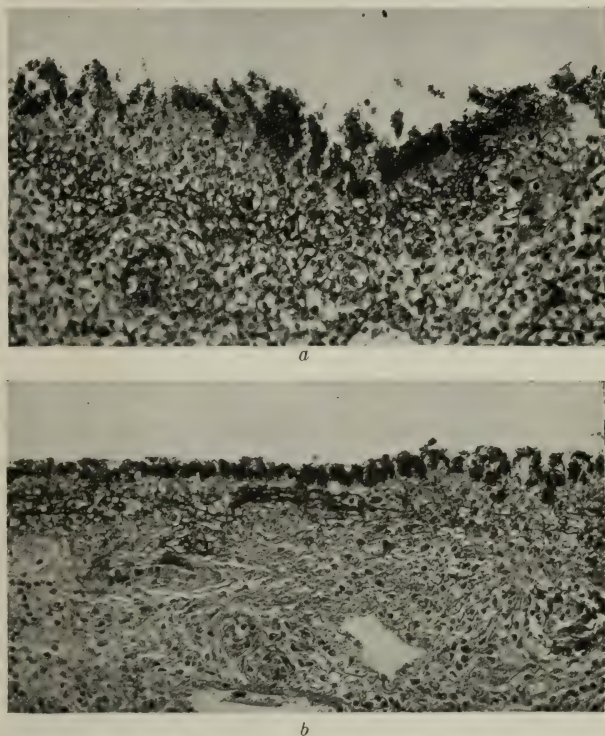
Fig. 71.—Scarlet fever—Gram-Weigert stain: *a*, Erosion on surface of pharynx, lined with masses of bacilli; considerable fibrin in inflammatory reaction; × 200; *b*, masses of bacilli in upper surface of membrane in larynx; × 200 (Mallory and Medlar).

Bacillus Mallei (Bacillus of Glanders).—*Morphology.*—
Bacilli of medium size, variable in length, having round or conical ends, and frequently showing faintly stained areas in their protoplasm (Fig. 72). The larger forms of the bacillus are usually slightly bent or wavy in outline. Slight irregu-

[1] Parker, *J. Med. Research*, 1922, xliii, 387.

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larities in shape may be observed. The morphology varies considerably in different culture-media.

In smear preparations from the lesions the bacilli usually appear somewhat longer and thicker than the tubercle bacillus,
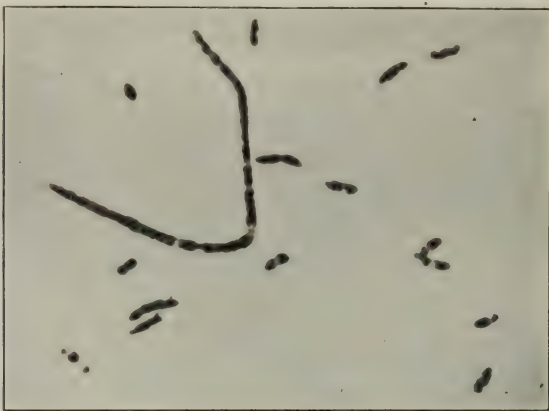
FIG. 72.—Glanders bacilli from a young culture on potato; × 2000 (Wright and Brown).

and show numerous sharply defined, unstained or faintly stained areas in their protoplasm (Fig. 73). They have rounded or conical ends, and are sometimes slightly irregular in shape. As a rule, they are present in small numbers. If Löffler's

FIG. 73.—Glanders bacilli in a smear preparation from a lesion in a guinea-pig, showing the marked irregularity in the staining of the bacilli; × 2000 (Wright and Brown).

methylene-blue solution is used for staining the smear, it should be heated; if carbol-fuchsin is used, it should be followed by a slight decolorization with 95 per cent. alcohol to better differentiate the bacilli. Gram-negative; non-motile.

Blood-serum.—Rounded, elevated, colorless, viscid-looking colonies, growing slowly and becoming well developed after thirty-six hours in the incubator. They may attain a diameter of 2 or 3 mm., and after a time they assume a brownish tint.

Potato.—After thirty-six hours in the incubator a rather thick, colorless, viscid-looking layer appears, which soon assumes a brownish tint and resembles honey in appearance. Later the brown color changes to a dark reddish-brown, and the growth becomes thicker and more opaque, while the potato takes on a dark gray color.

Bouillon.—Diffusely clouded, with the formation of a viscid sediment.

Litmus-milk.—Gradually turned red and coagulated.

Agar and Gelatin.—Growth not especially characteristic.

Pathogenesis.—When inoculated subcutaneously into male guinea-pigs, the characteristic results are swelling and inflammation of the scrotum, appearing after a variable number of days, often after a week.

The animals usually survive several weeks, with ulceration at the point of inoculation. The lesions produced consist in suppurative processes or abscess-formations in or about the testes, in the lymph-glands, in the anterior nares, about the joints, and in other situations, besides small grayish nodules or areas in the viscera—the so-called "glanders tubercles." The suprarenal glands usually show red areas, and they may be enlarged. On microscopical examination the small nodules as well as the extensive suppurative areas will be found to be composed of necrotic material containing leucocytes and fragments of chromatin. The distribution and extent of the lesions vary with each animal, but the involvement of the testis or its membranes is practically constant and pathognomonic of the bacillus of glanders. This involvement of the testis may consist, in early cases, in the presence of yellow foci in or about the tunica vaginalis, or in later cases the organ may show large yellow areas with purulent softening.

Intraperitoneal inoculation with virulent cultures may be followed by death within forty-eight hours, with fibrinous

exudate on the peritoneum in which minute grayish nodules are seen. The nodules are made up of a material which is apparently mainly dead or degenerated leucocytes and desquamated peritoneal endothelium, together with many chromatin fragments.

In these acute cases also microscopical examination of the spleen and liver may show the presence of small nodules identical in structure with those seen in the more chronic cases. For the purpose of producing with cultures the characteristic lesions of the testis or its coverings it is better to inoculate the animal subcutaneously, for in the rapidly fatal intraperitoneal inoculations with virulent cultures these may not show any marked changes.

The bacilli may be cultivated from the lesions, but not from the blood, in the chronic cases. They may be present in the blood, however, in small numbers in rapidly fatal infections following intraperitoneal inoculation.

Field-mice may die from subcutaneous inoculation in about seventy-two hours. The most conspicuous lesion produced is enlargement of the spleen, with the presence in it of minute grayish nodules. White mice are immune. Rabbits are not as susceptible as guinea-pigs to the infection.

Occurrence.—Found in the lesions of glanders and of farcy, and may invade the blood-stream in small numbers in acute cases of infection. Grows in the tissues in clumps or groups as well as scattered. In lesions on exposed surfaces it may be accompanied by the pyogenic cocci. We have succeeded in demonstrating the presence of the bacillus in the sputum of a case of human glanders by inoculation of a guinea-pig with the sputum.

Diagnosis.—In a case of suspected glanders the discharges from sinuses or ulcerated surfaces, or the contents of pustules, are to be examined for the presence of the bacillus of glanders by the usual methods.

The material for examination may be collected on "swabs." With this a guinea-pig is to be inoculated and cultures and smear preparations are made. If the material is from sinuses or ulcerated surfaces, the isolation of the bacillus by cultures

is difficult, owing to the presence of other organisms. The guinea-pig should be inoculated in the peritoneal cavity by introducing the infected swab into it through an incision in the abdominal wall, or by injecting about 1 c.c. of a suspension in bouillon of the suspected material into the peritoneal cavity with a hypodermic syringe.

If the bacillus of glanders is present, the scrotum will usually show the characteristic swelling and inflammation in the course of three or four days, and death will occur after some weeks. In some cases the animal may die in thirty-six or forty-eight hours. In any case the characteristic lesions of glanders will be found as described elsewhere, and the bacillus may be isolated from them by cultures. The spleen will practically always yield glanders bacilli in pure culture even if no macroscopical lesion can be made out.

In cultures the organism should show those characteristics of morphology, of culture, and of pathogenesis which have been described above.

The diagnosis of glanders in animals is facilitated by the use of mallein. Mallein is the name given to the endotoxin derived from the bacilli, and is prepared by allowing the organisms to grow in glycerine bouillon for a long period of time (one to five months). The subcutaneous injection of a proper dose of mallein into a horse or other animal suffering from glanders results in a marked rise of temperature ($104°–106°$ F.) developing in eight to ten hours with a gradual fall to normal in two or three days. There develops locally at the site of inoculation marked induration which may attain a diameter of 25 to 30 cm. and which is hot and extremely tender during the first twenty-four hours. In normal animals there is usually a slight rise in temperature ($101°–103°$ F.) which subsides in twenty-four hours, together with slightly elevated swelling (5–10 cm. in diameter) at the site of inoculation.

The serum of individuals suffering from glanders is able to agglutinate in high dilution the organism and to fix complement in the presence of antigen derived from the organism. Both properties are extremely useful diagnostically.

To Stain the Glanders Bacillus in Sections.—The bacilli are usually not numerous, and are scattered about in a mass of deeply staining fragmented nuclei, so that often they are recognized with great difficulty. The ordinary eosin-methylene-blue stain, after fixation in Zenker's fluid, can be highly recommended for demonstrating them. The following special methods have long been used for the same purpose.

Löffler's Methylene-blue Stain for Sections.—1. Stain paraffin sections twenty minutes in Löffler's methylene-blue solution or in equal parts of aniline-methyl-violet and 1:10,000 KOH solution.

2. Place for five seconds in the following solution:

Distilled water,	10 c.c.;
Concentrated sulphuric acid,	2 drops;
5 per cent. oxalic acid,	1 drop.

3. Wash out quickly in distilled water.
4. Absolute alcohol.
5. Xylol.
6. Xylol balsam.

It is recommended to place the section for a few minutes before staining in the 1:10,000 caustic-potash solution.

Schutz's Method.—1. Stain twenty-four hours in equal parts of concentrated alcoholic solution of methylene-blue and caustic potash, 1:10,000.

2. Wash in acidified water.
3. 50 per cent. alcohol for five minutes.
4. Absolute alcohol for five minutes.
5. Xylol.
6. Xylol balsam.

Noniewicz's Method.—1. Stain in Löffler's methylene-blue solution two to five minutes.

2. Wash in water.
3. Decolorize one to five seconds in

$\frac{1}{2}$ per cent. acetic acid,	75 parts;
$\frac{1}{2}$ per cent. aqueous solution of tropeolin,	25 "

4. Wash in water.
5. Dehydrate section on slide with filter-paper; then in the air; finally, over small flame.
6. Clear by dropping xylol on it repeatedly.
7. Xylol balsam.

Bacillus of Chancroid (Bacillus of Ducrey).—In smears from the lesions the bacilli appear as short, round-ended rods, about 1.5 μ long and 0.5 μ thick, occurring characteristically but not always in chains. The middle portion of the rods does not stain so deeply as the ends. The bacilli are decolorized by Gram's method of staining, and are not motile. The following description of the cultural peculiarities of the chancroid bacillus are based on the observations of Dr. Lincoln Davis in the Laboratory of the Massachusetts General Hospital.
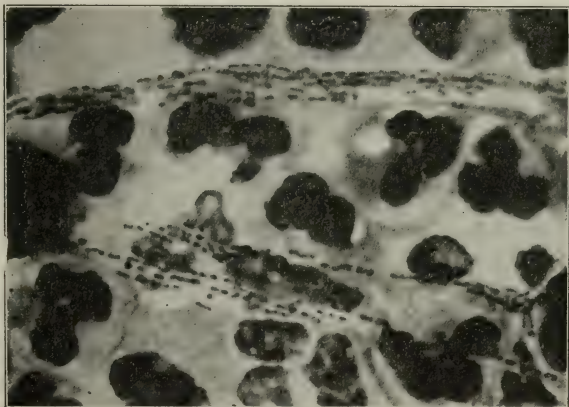
FIG. 74.—Bacillus of chancroid in smear preparation from pus (Lincoln Davis; photo. by L. S. Brown).

The bacillus does not grow on the ordinary culture-media, but may be cultivated in blood or in media containing one-third its volume of blood. In tubes containing blood, or a mixture of bouillon and blood, after twenty-four hours in the incubator, the growth appears as whitish flocculi at the bottom of the tube. These flocculi are composed of tangled chains of the bacilli, the chains being often of extreme length. The individual bacilli, as a rule, have the same morphology and staining reactions as in the smears from lesions, but occasionally long or even filamentous forms may be seen. On the surface of slant tubes, composed of a mixture of blood and agar, the bacillus forms, after forty-eight hours in the incubator, small, rounded, grayish colonies difficult to pick up with the platinum

wire, because they tend to glide before it. The bacilli from these colonies appear in smear preparations in short chains and singly. Involution forms are early apparent among the bacilli in all cultures. The bacillus dies out in cultures after about three days. Upon a monkey of the genus *Macaccus*, small ulcerations in the skin were produced by inoculation with cultures.
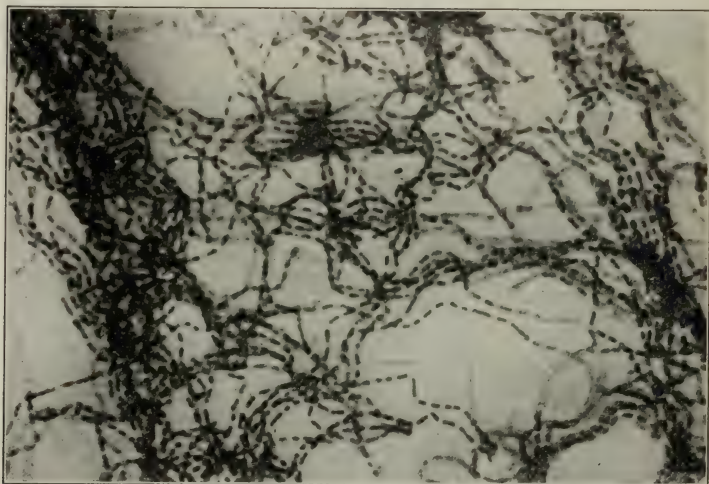
FIG. 75.—Bacillus of chancroid from culture (Lincoln Davis; photo. by L. S. Brown).

Method of Isolation from the Lesions.—Dr. Davis found that pure cultures were readily obtained by inoculating a small quantity of human blood in small tubes with material from the lesions, the blood apparently inhibiting or destroying other bacteria. These small tubes containing blood are most easily prepared in a way devised by J. H. Wright. A small glass tube, about 5 or 6 cm. long and 4 or 5 mm. in internal diameter, is drawn out into a fine caliber at one end, and is then sterilized throughout in the gas-flame. When cool, the pointed extremity of the tube is immersed in the blood obtained from a needle-prick in the skin of the dorsum of the thumb near the nail, and then by manipulation of the tube the blood is caused to flow into it. In this way a sufficient quantity—say, 0.2 to 0.5 c.c.—

is easily collected in the tube, after which the pointed end is sealed in the flame and the tube is ready to be inoculated. The other end of the tube is plugged with cotton, which is impregnated with paraffin to prevent evaporation. The skin, before being pricked, is sufficiently cleansed by soap and water, followed by alcohol. A small tourniquet is applied about the base of the thumb, to increase the flow of blood from the needle-prick.

Teague and Deibert[1] have recently devised a method for which they claim a diagnosis can be made in over 90 per cent. of true chancroids. Sterile rabbit blood is placed in 1 c.c. amounts in small test tubes, allowed to clot at room temperature, and either heated for five minutes at $55°$ C. for immediate use or allowed to stand for three or four days without heating at ice box temperature. Material is obtained from the lesions by means of short lengths of stiff iron wire bent back on themselves for about $\frac{1}{8}$ inch at the tip and sterilized in the hot air sterilizer. After incubation the separated serum is examined for the presence of the organisms.

Bacillus Pyocyaneus (Bacillus of Green Pus).—*Morphology.*—Small bacilli with rounded ends (Fig. 76).

FIG. 76.—Bacillus pyocyaneus showing flagella, from a preparation stained by Dr. Hugh Williams; × 2000 (Wright and Brown).

Decolorized by Gram's method (Welch). Motile, and is provided with a flagellum at one end. Does not form spores.

Blood-serum colonies grow rapidly, are not especially characteristic in form, and liquefy the medium, imparting to it a dark greenish color.

Gelatin Stab.—Liquefaction in funnel form, with green fluorescence of the upper portions of the medium. The liquefied

[1] Teague and Deibert, *J. of Urol.*, 1920, iv, 543.

gelatin is densely clouded, and there may be a viscid pellicle on the surface.

Agar Stab.—A green fluorescence in the upper layers of the medium, which later becomes a dark blue-green.

Potato.—Slightly elevated, brownish, viscid layer. The potato in some cases assumes a green color, in others a brown color. In some cultures the potato when touched with the platinum wire takes on a green color at the point touched. This is the so-called "chameleon phenomenon," and it is best observed in cultures several days old.

Bouillon.—The growth is in the form of flocculi and a delicate surface pellicle. The fluid acquires a green color.

Litmus-milk.—Acid reaction with coagulation.

Dunham's Pepton Solution.—Indol is produced.
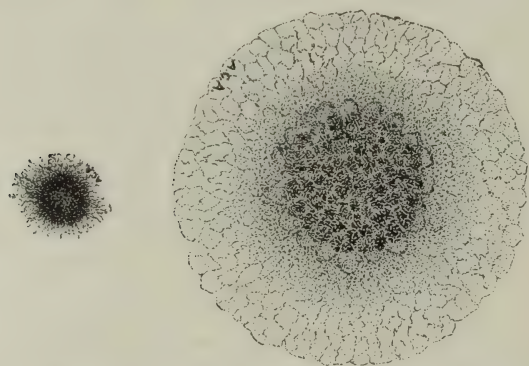
Fig. 77.—Bacillus pyocyaneus: colonies upon gelatin (Abbott).

Colonies on Gelatin Plates (Fig. 77).—Development is rapid. Young colonies are provided with a fringe of delicate filaments about their periphery. As growth progresses and liquefaction becomes more advanced, the central mass of the colony sinks into the liquefied depression, while at the same time there is an extension of the colony laterally. . . . At this stage the colony, when slightly magnified, may present various appearances, the most common being that shown in Fig. 77. The gelatin between the growing colonies takes on a bright yellowish-green color, but, as growth is comparatively rapid, it is quickly

entirely liquefied, and one often sees the colonies floating about in the pale-green fluid.

Pigments.—Two pigments are produced by the bacillus. The first, "pyocyanin," may be extracted from cultures by chloroform and on evaporation separates out as blue stellate crystals. The second pigment is fluorescent, insoluble in chloroform, soluble in water, and of the sort common to other fluorescent bacteria.

Toxin Production.—It is claimed that the bacilli produce a true exotoxin, very much weaker than either diphtheria or tetanus toxin.

Pathogenesis.—Subcutaneous inoculation of guinea-pigs and rabbits with 1 c.c. of a virulent bouillon culture may produce purulent infiltration and inflammatory edema of the tissue about the point of inoculation, and death may follow in eighteen to thirty-six hours. Intraperitoneal inoculation may result in a sero-fibrinous or purulent peritonitis with fatal result. In fatal inoculations the bacillus is found in the various viscera, but not in great numbers. Animals inoculated with small amounts may survive with merely local lesions, and an immunity may be produced.

Several varieties of this bacillus have been described, but their differences do not seem to be of sufficient importance to justify their separation into distinct species.

Occurrence.—"Is widely distributed, occurring often on the human skin, in the feces, and outside of the body. In wounds, stains the dressings bluish-green and produces a somewhat characteristic offensive odor.

"Increases suppuration of wounds, usually with little constitutional disturbance. Is found not infrequently in perforative peritonitis and appendicitis, sometimes in phlegmons, otitis media, broncho-pneumonia, and inflammation of serous membranes, associated usually with other bacteria.

"It was found by H. C. Ernst in tuberculous pericarditis. Often found in diarrheal and dysenteric discharges. May cause general infection in human beings. With or without general infection it may cause hemorrhagic and necrotic enter-

itis, a form of pyocyaneous infection in human beings which we have repeatedly observed at autopsy. Instances of invasion of the body from wounds by the bacillus pyocyaneus have not been observed '' (Welch).

In general it may be said that Bacillus pyocyaneus is an organism of low virulence, that it is most commonly present in pathological lesions as a secondary invader, and that when it does assume the rôle of a primary invader it is usually in an individual of lowered vitality.

Bacillus Pestis.—*Morphology.*—In the tissues the organism occurs as a medium-sized short bacillus with rounded ends.
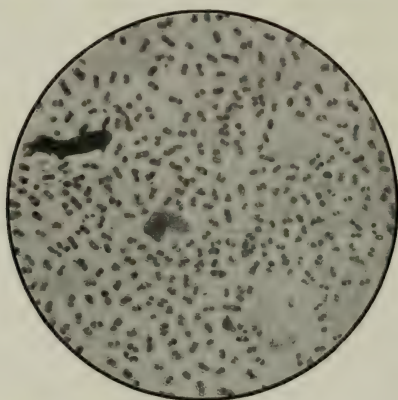
Fig. 78.—Bacillus pestis (Yersin).

In cultures its size and length vary and its median portion may be swollen so that an ovoid form is produced; it may grow in pairs and in chains, and it may occur as long, thread-like forms. Involution forms of elliptical or round shape, and often of large size, sometimes resembling yeast-cells, are frequent in old cultures or in cultures on special media. These involution forms are easily produced by cultivation on agar containing 3 to 4 per cent. of sodium chlorid.

Staining.—The organism stains with the usual aniline dyes, and is decolorized by Gram's method of staining. In the tissues it stains more deeply at its extremities than at its central portions, and it sometimes appears to possess a capsule. The

polar staining may sometimes be brought out in cultures by weak staining solutions or by decolorization by alcohol. It is not motile, and it does not form spores.

Gelatin Plates.—The colonies on the surface appear after twenty-four to forty-eight hours at 22° C. They are flat, round, and white or yellowish white in color. Under a low magnifying power the central portion of the colony is granular, while the marginal portion is clear. The colonies do not spread over the surface of the medium.
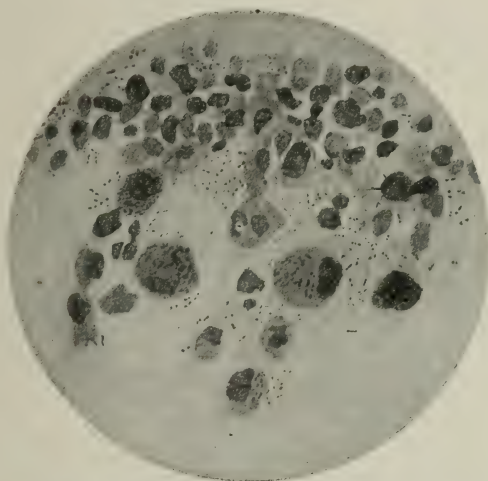
FIG. 79.—Bacilli of plague and phagocytes, from human lymph node; × 800 (Aoyama).

Gelatin Stab.—Growth all along the line of inoculation with the formation of a layer of growth at the surface of a whitish color. There is no liquefaction of the gelatin.

Gelatin Slants.—A whitish or slightly yellowish layer presenting nothing characteristic.

Agar Plates.—The colonies on the surface appear first as dew-drops, and have already attained their maximum development after twenty-four to forty-eight hours in the incubator. They will then grow white in color, and present an opalescent or iridescent margin. Under the microscope they are distinctly granular. Considerable difference in size may be observed

among the colonies. The larger colonies are said to be less virulent for animals than the smaller colonies, and it is claimed that these larger colonies when transplanted give rise to large colonies again.

Bouillon.—The fluid usually remains clear, and the growth appears in the form of a granular or flocculent sediment which may here and there adhere to the wall of the tube. In bouillon cultures richly inoculated and retained in a perfectly upright and undisturbed position at room-temperature for some days a characteristic appearance is produced. In twenty-four to forty-eight hours islands of growth appear underneath the surface in the form of flakes. In the next twenty-four to forty-eight hours there grow down into the fluid from the flakes long, stalactite-like masses, the liquid remaining clear. In four to six days the islands of growth have become more compact and solidified. If the flask be now slightly disturbed, the islands fall to the bottom, bringing with them the stalactite-like growths. The latter are very fragile. In addition to these appearances there is a deposit of growth on the wall of the flask and at the bottom, as well as a ring of growth on the margin of the surface of the liquid.

Milk.—Growth without coagulation.

General.—No production of indol. In neutral litmus bouillon the blue color is changed to red. There is no odor, and no pigment production.

The organism is aërobic. It remains alive in cultures for five to six weeks at least. Growth occurs at all temperatures from 4° to 37° C. The best temperature for growth is 30° to 32° C.

Pathogenesis.—The organism is pathogenic for a great variety of animals, including mice, rats, guinea-pigs, rabbits, the California ground squirrel, and other rodents. In these animals death generally follows in from two to six days after subcutaneous inoculation. The lesions produced are hemorrhagic edema at the seat of inoculation, enlargement of the lymph nodes with more or less hemorrhage, enlargement of the spleen and its follicles. The bacilli are present in large numbers

in the enlarged lymph nodes and in the internal organs; they are less numerous in the blood. Pigeons, chickens, and cattle are immune.

Occurrence.—The bacillus is found in large numbers in the buboes, pustules, pulmonary lesions, and other localized lesions of the bubonic plague. It also may be found in larger or smaller numbers in the blood and internal organs generally, and it may be present in the sputum, bile, and alvine discharges. The pus of the buboes which break spontaneously may be sterile.

Bacteriological Diagnosis.—In cases of suspected plague the bacillus is to be sought for in the blood and in the buboes. In cases of pneumonia the sputum especially is to be examined. In the examination cultures as well as smear preparations are to be used.

Perhaps the most certain method of identification of the bacillus is the inoculation of the mucous membrane of the nose of the rat. The simple rubbing of a portion of the culture upon the mucous membrane appears to be sufficient to produce a fatal result in the rat if the culture contains the genuine bacillus. As a culture-medium agar or blood-serum is to be used in cases where there is no mixed infection. If there is mixed infection of the material to be examined, gelatin plate cultures are to be made. The vigorous rubbing of cultures on the unbroken skin of guinea-pigs will likewise result in infection.

The inoculation of animals for diagnostic purposes should be made with the greatest precaution to prevent the spread of the disease.

Bacillus Anthracis.—*Morphology.*—The organism grows in long segmented threads, the segments varying in length, but usually being two or three times as long as broad and having square or slightly concave ends. These segments represent the bacillus, which is among the largest of the bacteria (Fig. 84).

Stained by Gram's method. Not motile.

Forms oval spores in the middle of the short segments or rods. The spores may be seen in blood-serum cultures after forty-eight hours in the incubator (Fig. 80).

Blood-serum.—Irregularly rounded colonies, several milli-meters in diameter after twenty-four hours in the incubator.
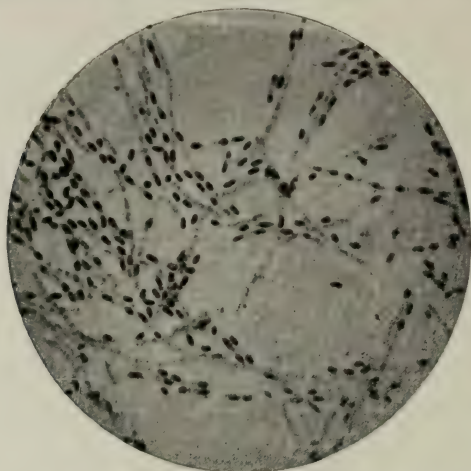
FIG. 80.—Bacillus of anthrax, stained to show the spores; × 1000 (Fränkel and Pfeiffer).

The colonies are grayish, finely granular, and have the appearance of being made up of a dense network of delicate fibrillæ. The blood-serum is slowly liquefied.

FIG. 81.—Bacillus of anthrax: gelatin stab-culture seven days old (Günther).

Gelatin Stab.—Growth along the line of stab, with radiating filaments extending laterally into the gelatin, which is slowly liquefied in funnel form (Fig. 81).

Bouillon.—Growth in the form of cotton-like flakes and filamentous masses. No clouding of the medium.

Agar.—Matted network of translucent filaments. Under a
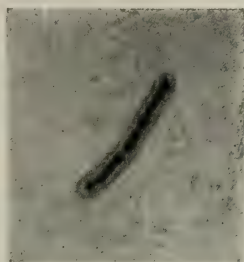
Fig. 82.—Bacillus of anthrax; smear preparation from vesicle. Stained by W. H. Smith's method; × 1800 (W. H. Smith; photo. by L. S. Brown).

lower magnifying power the growth is seen to be made up of twisted and contorted masses of filaments, giving the appearance of curled hair (Fig. 83).
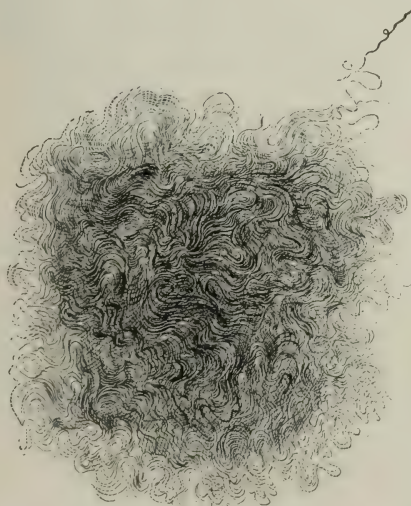
Fig. 83.—Colony of bacillus of anthrax, slightly magnified (Flügge).

Potato.—Grayish-white, rather thick, dry layer, having the appearance of frosted glass.

Pathogenesis.—Mice, guinea-pigs, and rabbits inoculated subcutaneously die with a general invasion of the blood by

24

the organism. Mice are most susceptible to the infection, dying in about twenty-four hours, while guinea-pigs and rabbits survive longer.

In all these animals the most striking lesion is a large soft spleen, and in the guinea-pig also an extensive inflammatory edema of the subcutaneous tissues. On microscopic examination the bacilli will be found in the organs and blood. If,
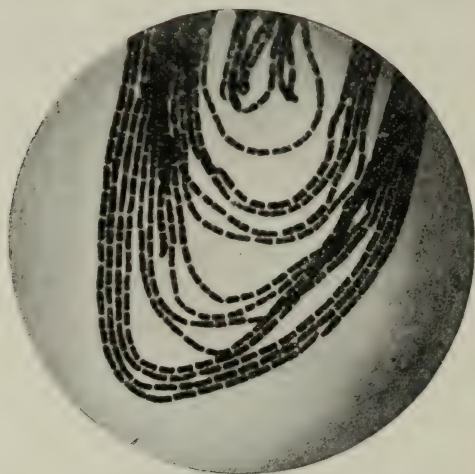
FIG. 84.—Bacillus of anthrax: portion of a colony three days old upon a gelatin plate; × 1000 (Fränkel and Pfeiffer).

the animal has been dead some time, the number of bacilli present in these situations will be very great, owing to the post-mortem growth. It is characteristic of the bacillus of anthrax in smear preparations from infected tissues that it should have a narrow capsule (Fig. 85) and show square or slightly concave ends. The capsule is not present in cultures.

Occurrence.—In malignant pustules, wool-sorter's disease, and intestinal anthrax. Found in the blood of animals dead of anthrax. In man the infection is usually localized at first at the point of inoculation, either on the skin or on the mucous membrane of the air-passages or intestinal tract. Later, a general invasion of the blood may occur and a fatal

septicemia result. The organism or its spores may be present in wool or hides, and infection may take place from these.

Diagnosis.—The bacilli may be found by the smear examination of the contents of the small blebs and vesicles. The bacillus of anthrax may be identified by its morphology (see p. 367), its special characteristics being its large size and its square or concave extremities.
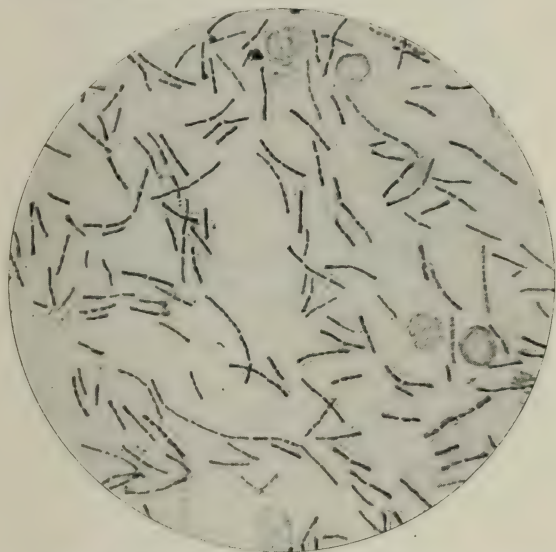
FIG. 85.—Bacillus of anthrax from spleen of a mouse (L. Frothingham).

The inoculation of a mouse at the root of the tail with some of the material from the pustule, and the production of the characteristic fatal septicemia, will render the identification certain.

Bacillus Mucosus Capsulatus.—*Synonym.*—Friedländer's bacillus.

Morphology.—Bacilli of moderate size, usually two or three times as long as broad, with rounded ends, occurring frequently in pairs and sometimes in long forms. Occasionally in cultures it shows a wide capsule. The capsule, however, is best shown in smear preparations from infected tissues (Figs. 86, 87). Gram-negative; not motile.

Blood-serum.—After twenty-four to thirty-six hours in the incubator the colonies appear as translucent, colorless, rounded,
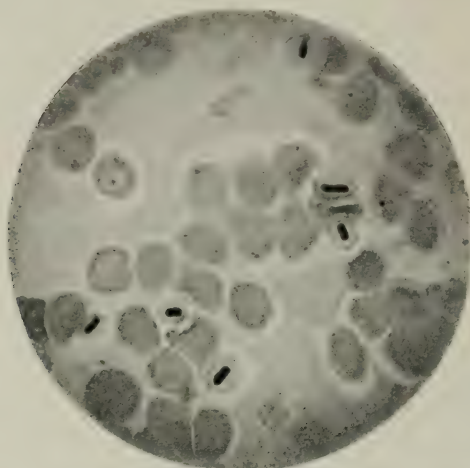
Fig. 86.—Bacillus mucosus capsulatus in blood; × 1000 (Fränkel and Pfeiffer).

convex elevations, resembling drops of mucus.   If few in number, they may attain a diameter of 2–3 mm.   They are viscid, and when touched with the platinum wire may be drawn out into
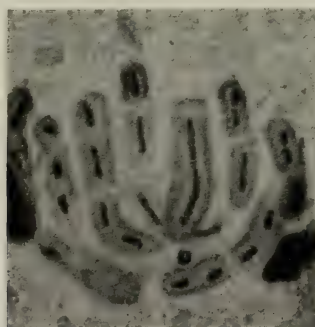
Fig. 87.—Bacillus mucosus capsulatus; smear preparation from sputum. Stained by W. H. Smith's method; × 1500 (W. H. Smith; photo. by L. S. Brown).

threads.   The water of condensation may become thick or viscid from the growth of the organism in it.

Plain and Blood Agar.—Colonies similar to those on blood-serum.   They show a marked tendency to coalesce.

Dextrose Agar Stab.—Growth along the line of inoculation, with the production of a few gas-bubbles in the medium.

Bouillon.—Clouded with the formation of a thin pellicle.

Potato.—Thin, colorless, viscid layer.

Litmus-milk.—Turned red and coagulated.

Gelatin.—Growth not remarkable

There apparently exists a number of varieties of aërobic capsulated bacilli differing from one another only in non-essential particulars.   The organism here described is to be taken as a type of a group of closely-related bacteria of which *B. mucosus capsulatus* is a well-known member.

Pathogenesis.—White mice, rabbits, and guinea-pigs die from septicemia in a short time after inoculation, the encapsulated bacilli being present in the organs and blood of the heart in large numbers.

White mice die in twenty-four hours to three days.   Rabbits inoculated in the ear vein and guinea-pigs inoculated in the peritoneal cavity may die within twenty-four hours.

Subcutaneous inoculation of the animals last named leads only to local suppuration.   The lesions produced by this organism consist in marked congestion of the superficial veins, hemorrhage into the lymphatic glands, and enlargement and softening of the spleen.   In the guinea-pig a hemorrhagic condition of the suprarenal capsules is present, and in the peritoneal cavity there may be a small amount of clear, rather viscid fluid containing the bacilli in large numbers.

The organs on microscopic examination may show peculiar areas in which the cells and nuclei are shrunken and in which the bacilli are aggregated.

Occurrence.—This organism or closely related forms may be met with in broncho- or lobular pneumonia and in inflammatory conditions of the air-passages generally.   It may also be present in the upper air-passages of healthy individuals. It has been observed in inflammations of the middle ear, in empyema, meningitis, endocarditis, peritonitis, and in pus

formations. In fatal infections the blood-stream may be found invaded by the organism. It is held by some bacteriologists that the members of this group may be the infective agents in genuine lobar pneumonia in rare instances. Representatives of this group have been found in the soil, in the air, and in contaminated water.
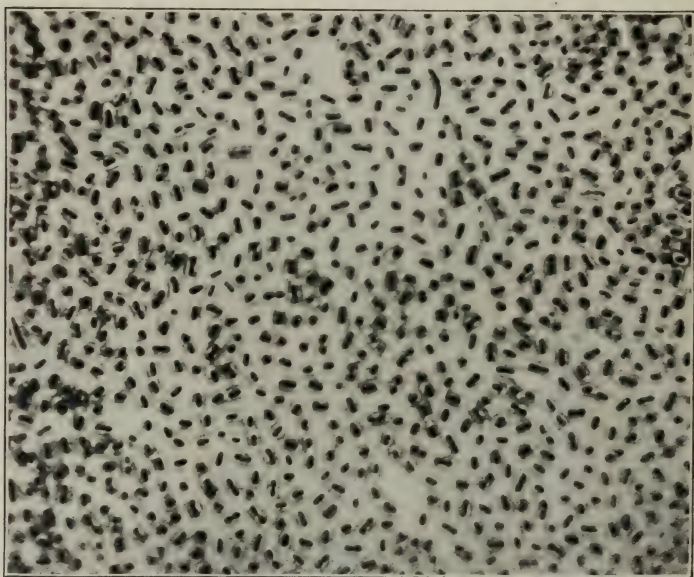
FIG. 88.—Bacillus mucosus capsulatus, from a culture; × 1000 (Wright and Brown).

Method for Staining Capsules in Sections.—1. Stain for twenty-four hours in the incubator in the following solution:

Concentrated alcoholic solution of methyl-violet, 50;
Distilled water,   100;
Glacial acetic acid,   10.

2. Wash out in a 1 per cent. solution of acetic acid.
3. Alcohol.
4. Oil.
5. Xylol balsam.

If the process of decolorization is stopped at the right moment, the capsules will be pale blue, while the bacilli will be stained deep blue.

Bacillus of Rhinoscleroma.—The organism belongs to the same group as Bacillus mucosus capsulatus and closely resembles the latter morphologically and culturally. It differs in that it forms no gas in media containing dextrose, does not form acid with lactose, and never coagulates milk.

Method of Staining Capsules in Sections of Tissues Hardened in Alcohol (Wolkowitsch).—1. Stain twenty-four to forty-eight hours in aniline-methyl-violet.

2. Wash off in water.

3. Iodin solution one to four minutes.

4. Absolute alcohol.

5. Oil of cloves, which removes still more of the color.

6. Xylol.

7. Xylol balsam.

According to Wolkowitsch, the hyaline masses in rhinoscleroma stain intensely with methyl-violet, gentian-violet, methylene-blue, and fuchsin; less with safranin, and not at all with hematoxylin. Eosin stains them well. Double staining with hematoxylin and eosin is therefore to be recommended highly.

Bacillus Welchii (B. Aërogenes Capsulatus) and Related Anaërobic Organisms.—Prior to the World War very little was known about the bacteriology of the so-called "gas bacillus" infections. It was generally assumed that such infections were most commonly caused by B. Welchii, although various other anaërobic organisms had been isolated here and there by different investigators. Since 1914 a great deal of work has been reported relative to the bacteriological findings in the thousands of "gas bacillus" infections occurring during the war. It is not within the scope of this book to go into details concerning the various organisms isolated. A brief summary only will be given. For more complete knowledge the reader is referred to Weinberg and Séguin "La Gangrène gazeuse," Masson et Cie., 1917, and to Report No. 1 of

the Anaërobic Committee of the Medical Research Committee of Great Britain, April, 1918. Excellent summaries appear in Hiss-Zinsser and Russell "A Textbook of Bacteriology," Appleton, 1922, and in "Laboratory Methods of the U. S. Army," Medical War Manual, No. 6.

All of the organisms of this group are habitants of the normal gastro-intestinal tract, and wound infection primarily demands infection directly or indirectly with fecally contaminated material (usually soil). It is generally considered that all these organisms are saprophytes, *per se*, hence non-invasive, and are harmful only because of the powerful toxins which they produce. For growth a condition of relative anaërobiosis and the presence of devitalized tissue are required. Indirectly they can be said to be invasive for their toxins destroy contiguous healthy tissue and thus furnish a favorable medium for spreading growth. The anaërobic organisms isolated from a case of "gas bacillus" infection or "gas gangrene" rarely consist of only one strain. More commonly two or more strains are found together with various aërobic organisms, such as, staphylococci, streptococci, and B. pyocyaneus.

The anaërobic bacilli may be divided into two groups—saccharolytic and proteolytic. The former includes B. Welchii, Vibrion septique, B. edematiens, and B. fallax. The latter includes B. sporogenes, B. histolyticus, and B. putrificus. The saccharolytic group do not liquefy coagulated horse serum, but form acid and gas in milk. With the exception of B. fallax they produce powerful soluble toxins. The proteolytic group liquefy coagulated horse serum and digest milk with the formation of alkali. They do not produce soluble toxins, and are important only because by their growth and consequent tissue lysis they furnish a favorable medium for the members of the saccharolytic group.

The morphological, cultural, and biological characteristics of the more important members of this group follow:

B. Welchii.—Short, fat, Gram stained bacillus usually with slightly rounded ends and frequently showing a capsule (Fig. 89). Occurs singly and in pairs, rarely in short chains, non-

motile. Does not form spores in the presence of fermentable sugars. Spores large, oval, and central or subterminal. In milk produces "stormy fermentation" with the formation of lactic and butyric acids. This rapid acid clot torn apart by gas bubbles is so characteristic as to be considered diagnostic for B. Welchii. Ferments all the common sugars, but does not ferment salicin. Grows best under conditions of strict anaëro-biosis, but growth will occur under conditions of diminished oxygen tension. Extremely pathogenic for guinea-pigs and pigeons; less pathogenic for rabbits and mice. It produces a
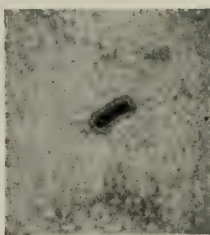
FIG. 89.—Bacillus Welchii; smear preparation from the spleen. Stained by W. H. Smith's method; × 1500 (W. H. Smith; photo. by L. S. Brown).

powerful soluble toxin, against which a potent antitoxin can be formed by the injection of suitable animals. The antitoxin protects laboratory animals from otherwise fatal doses of the toxin.

Vibrion septique.—Rather slender, Gram stained, motile bacillus with slightly rounded ends. Club-shaped, small oval, and irregularly stained and shaped forms, if present, are characteristic. Strict anaërobe. No capsules. Central or sub-terminal spores are readily formed in most media. Ferments the common sugars including salicin, with the exception of saccharose. In milk it produces acid and clot in two to five days with often some gas formation. Liquefies gelatin. Hemolytic.

It is pathogenic for guinea-pigs, rabbits, mice, and pigeons. It may be recovered from the blood at the time of death. The livers of guinea-pigs which have died show long filamentous forms—a finding so characteristic as to be diagnostically of great importance. Powerful soluble toxins are produced by all

strains.   Antitoxins may be prepared which in high dilution protect against an otherwise fatal dose of the organisms.

B. edematiens.—Large Gram stained bacillus with square ends.   Practically   non-motile.   Strict   anaërobe.   Forms chains in cultures.   Curved forms often present.   Oval subterminal spores formed in all media.   Ferments glucose, levulose, and maltose, but does not ferment saccharose or salicin. Acid clot in milk after several days.   Usually pathogenic for guinea-pigs and mice.   Produces a colorless gelatinous edema
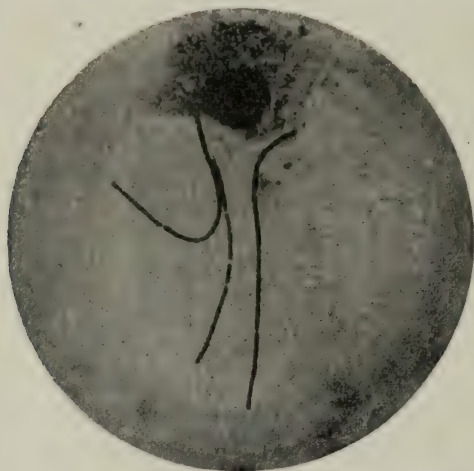
FIG. 90.—Bacillus of malignant edema (probably identical with B. sporogenes) from the edema fluid of a guinea-pig inoculated with garden-earth; × 1000 (Fränkel and Pfeiffer).

and may invade the blood stream.   Soluble toxins are formed, and antitoxins may be prepared.

B. sporogenes.—Motile, Gram stained, anaërobic bacillus. Forms oval subterminal spores in all media and the animal body. Usually non-hemolytic.   Markedly proteolytic.   Digests and blackens meat.   In milk causes precipitation of casein which is rapidly digested with separation of slightly yellow turbid whey. Is not pathogenic for the ordinary laboratory animals.   Does not produce a soluble toxin.   This organism usually is the cause of foul smelling wounds.   It is probably identical with Koch's bacillus of malignant edema (Figs. 90, 91).

B. histolyticus.—Motile, Gram stained, anaërobic bacillus with rounded ends which produces large oval terminal spores. Non-hemolytic. Markedly proteolytic. Non-pathogenic. No soluble toxin produced.

Isolation and Diagnosis.—The bacteriological diagnosis of infection due to one or more of these anaërobic organisms is of the utmost practical importance as the surgical treatment is usually changed accordingly. The presence of Gram stained bacilli in smear preparations should be considered indicative of such infection. The following method will serve to give a general idea as to the presence of the more important pathogenic organisms of this group:

The suspected material should be inoculated into two tubes of litmus milk overlaid with vaseline. The tubes prior to inoculation should be heated to 100° C. and rapidly cooled. "Stormy fermentation" in twenty-four to forty-eight hours suggests the presence of B. Welchii. Heat one tube at 60° C. for thirty or forty minutes and the other at 80° C. for twenty minutes. The former will suffice to kill the majority of the aërobes, and the latter will kill aërobes, and usually B.

FIG. 91.—Bacillus of malignant edema (probably identical with B. sporogenes); colonies growing in dextrose-gelatin (Fränkel and Pfeiffer).

Welchii and other non-sporing bacilli. Inoculate from each to a second tube of litmus milk. "Stormy fermentation" in the sub-culture from the tube heated to 60° C. suffices for the diagnosis of B. Welchii, providing bacilli of correct morphology can be demonstrated in stained preparations. This may be confirmed by injecting 0.5 to 1.0 c.c. of the culture intravenously into a rabbit. The animal should be killed in five minutes and placed

in an incubator for five or six hours. The presence of B. Welchii
is indicated by marked gaseous distension, subcutaneous crepita-
tion, and the finding of gas bubbles throughout the organs, partic-
ularly in the liver. Smear preparations show enormous numbers
of bacilli of typical morphology.   Growth in the subculture
from the tube heated to 80° C. indicates the probable presence
of one or more species which produce spores in milk, of which
the most likely are Vibrion septique, B. edematiens, B. sporo-
genes or B. histolyticus.   Hanging drop preparations should
be examined for motility.   If Vibrion septique is present, 1 c.c.
injected into a guinea-pig intramuscularly will cause death
within twenty-four hours with the characteristic findings—
blood stained edema, intense red coloration of affected muscles,
and filamentous forms of the organism in film preparations
from the peritoneal surface of the liver. Whitish gelatinous
edema and the absence of filamentous forms from the surface of
the liver are characteristic of death due to B. edematiens.

If the original milk tubes do not show stormy fermentation
at the end of forty-eight hours it may be concluded that B.
Welchii is not present.   If smear preparations made at this
time reveal Gram stained bacilli the cultures should be heated
and the subsequent steps taken as above.   No attempt has
been made to describe a method for isolating the proteolytic
members of this group as they are of little practical impor-
tance.   Pure cultures for final identification are best obtained by
anaërobic plate methods.

For efficacious serum treatment early diagnosis is of the
utmost importance.   With this in mind Henry and Lacy[1] have
suggested the following method.   Inoculate suspected material
into cooked meat medium[2] and incubate overnight.   From the
supernatant fluid inoculate a milk tube and two guinea-pigs, one
of which has received B. Welchii and Vibrion septique antitoxin
and the other B. Welchii and B. edematiens antitoxin.
"Stormy fermentation" in the milk tube indicates B. Welchii,

[1] Henry and Lacy, J. Path. and Bact., 1920, xxiii, 281.

[2] Cooked meat medium: Equal amounts of beef heart, freed from fat and
minced, and water.   Cook slowly.   Neutralize until alkaline to litmus.   Tube,
cover with vaseline and autoclave.

the death of the first pig B. edematiens and the death of the second pig Vibrion septique.

Bacillus of Tetanus.—In pure culture this bacillus will not grow in the presence of oxygen.

Morphology.—Slender rods with rounded ends, which may grow into long threads. In the incubator spores are rapidly formed. These are round, wider than the bacillus, and are situated at the end of the rod, giving the appearance of a
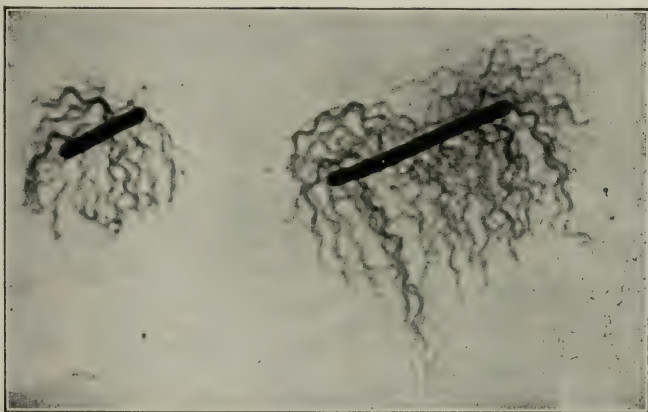
FIG. 92.—Tetanus bacilli showing flagella, from a preparation stained by Dr. Hugh Williams; × 2000 (Wright and Brown).

drum-stick or a round-headed pin (Fig. 96). Gram-positive; motile.

The *colonies* in anaërobic dextrose-gelatin cultures appear after several days as small clumps of interlacing fibrillæ from which delicate filaments radiate into the gelatin, which is slowly liquefied.

The colonies in simple anaërobic dextrose-agar plate cultures appear after twenty-four to forty-eight hours in the incubator, as groups and masses of long filaments radiating from a center (Fig. 93).

Dextrose-gelatin Stab.—Growth along the line of inoculation, beginning 2 or 3 cm. below the surface, with delicate filaments radiating laterally into the gelatin (Fig. 95). Liquefaction and gas-production occur.

In deep-stab cultures in slightly alkaline dextrose-agar (see Fig. 94) growth appears first all along the line of inoculation to within about 1 cm. of the surface after about twenty-four hours in the incubator. Later, lateral outgrowths extend into the medium from all along the line of inoculation below a point about 1 cm. below the surface. In the portion of the line of inoculation above this, growth is frequently observed up to the surface, but without lateral outgrowths. The growth eventually assumes the appearance of an inverted pine tree. A pecul-
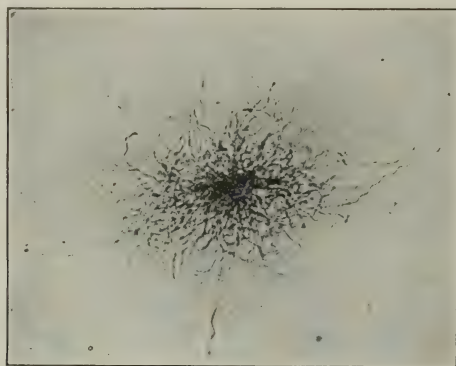
FIG. 93.—Colony of tetanus bacilli in anaërobic dextrose-agar plate; low magnifying power (Wright and Brown).

iar feature of the culture is the appearance of a brown pigmentation in the culture-medium in its upper layers in the form of a flat or cone-shaped zone. A small quantity of gas may be produced.

If the agar is slightly acid in reaction growth appears along the line of inoculation and spreads through the medium as a cloudiness extending to within a few millimeters of the surface. The employment of dextrose culture-media not older than a week or so seems to be important for success in cultivating this organism.

Dextrose-bouillon.—Growth appears first, after twenty-four to forty-eight hours, as a diffuse cloudiness. Later the fluid becomes clear, and a grayish sediment collects at the bottom of the tube. Only a small amount of gas is produced.

Pathogenesis.—Subcutaneous inoculation of mice at the root of the tail gives rise to tetanic symptoms in twenty-four hours, followed by death in two or three days.

Guinea-pigs and rabbits are also susceptible to the infection, the period of incubation in these animals being twenty-four to thirty hours in the former and two to three days in the latter animal, after subcutaneous inoculation. The symptoms of

FIG. 94.—Tetanus bacillus. Stab-culture in dextrose-agar. In the upper layers of the medium the peculiar brownish coloration is shown.

FIG. 95.—Bacillus of tetanus: six-days-old stab-culture in dextrose-gelatin (Fränkel and Pfeiffer).

tetanus appear first in the extremities nearest the point of inoculation. In mice the hind legs become rigidly extended backward. At the autopsy the bacillus is to be found only at the point of inoculation, and may be difficult or impossible to demonstrate there.

Occurrence.—Found in soil, and often in the feces of herbivorous animals. In cases of tetanus the bacillus is usually

found only in the wound or at the point of inoculation. Some workers have been able to demonstrate the organism in the blood stream and in various organs.

Toxin Production.—The bacillus of tetanus acts by the local production of a very powerful soluble toxin or toxalbumin. This is also produced in cultures. It may be demonstrated in the bacteria-free filtrate of bouillon cultures some days or weeks old. As little as 0.000,005 c.c. of such a filtrate may give rise to fatal intoxication in a mouse. Antitoxins can be produced of which 1.0 c.c. will protect against several hundred fatal doses of the toxin.
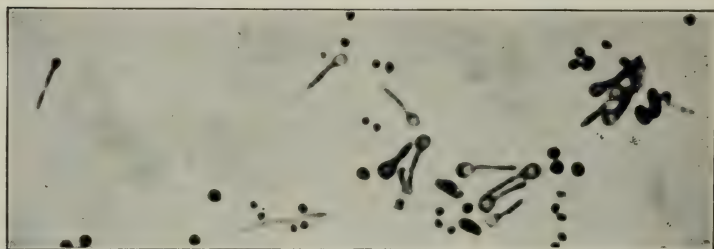
Fig. 96.—Spore-bearing tetanus bacilli in an impure culture on blood-serum from a case of tetanus. In the bacillus on the extreme left the beginning of spore-formation is shown (Wright and Brown).

Diagnosis.—The bacteriological diagnosis of tetanus is usually only confirmatory of the clinical diagnosis. The suspected material is obtained after the appearance of symptoms and, by the time a definite bacteriological diagnosis can be made the clinical symptoms are marked, providing tetanus antitoxin has not been used effectually. The appearance of typical sporing forms in smear preparations of the original material or cultures therefrom warrants a provisional diagnosis of tetanus.

Isolation.—Tetanus bacilli will grow in aërobic cultures if other bacteria are growing with them. Since tetanus wounds usually contain other bacteria, all that is necessary to obtain an impure culture of the tetanus bacillus is to inoculate an ordinary blood-serum or agar slant with material from the wound. After several days or a week in the incubator, if teta-

nus bacilli are present they can be recognized by smear preparations from the growth in the tube by their morphology and spore-formation (see Fig. 96). There will also be a peculiar, stinking odor about the culture. The isolation of the tetanus bacillus is now to be proceeded with as follows: Mix a loopful of the mixed growth on the blood-serum or agar with a tube of sterile bouillon, and heat in a water-bath for at least fifteen minutes at 80° C., then make anaërobic cultures from this, taking several loopfuls for inoculation.

If other spore-bearing bacilli are present in the mixed culture, it will be necessary to use some form of anaërobic culture on a solid medium in order to obtain separate colonies of the tetanus bacillus for further cultures.

The bacillus may be isolated from wounds and from the soil by inoculation of mice subcutaneously, and proceeding as above described with material from the seat of inoculation.

Bacillus Botulinus.—*Morphology.*—Large straight Gram stained bacillus with rounded ends usually single but occasionally occurring in short chains. Slightly motile. Subterminal, rarely central, oval spores readily formed in a favorable medium (alkaline dextrose gelatine) incubated at 20°–25° C.

It is a strict anaërobe. Grows readily on ordinary media if the reaction is neutral or slightly alkaline. Optimum temperature for growth 25° C. All cultures possess a peculiar odor similar to that of butyric acid.

Gelatin and Plain Agar.—Colonies round, opalescent, and yellowish with finely fringed edges. Gelatin liquefied.

Dextrose Agar Stab.—Growth first noticed as a narrow white column in the lower part of the tube. Later the agar is fragmented by gas bubbles.

Dextrose Bouillon.—General clouding with abundant gas formation.

Milk.—Not coagulated.

Toxin Production.—The organisms produce a powerful soluble toxin or exotoxin in any media, artificial or otherwise, in which they are able to grow. As little as 0.000,001 may kill a 250 gram guinea-pig in four days. By the injection of small

25

doses of toxin into susceptible animals it is possible to produce very potent antitoxin.

Pathogenesis.—Monkeys, guinea-pigs, and mice are very susceptible to the toxin; rabbits, cats, dogs and rats much less so.

Occurrence.—Has been recovered from various sources in nature, and is probably quite common. Occurs fairly frequently in canned and pickled food stuffs. The human ingestion of food so contaminated results in symptoms of severe intoxication and in the majority of cases (about 64 per cent.) death. The organisms may be recovered from the gastro-intestinal tract in cases which come to autopsy.

Isolation and Diagnosis.—Burke recommends direct inoculation of anaërobic agar shake cultures providing there are but few other organisms. In the presence of many contaminators the material should be generously inoculated into beef infusion broth containing 1 per cent. sodium chloride, 1 per cent. peptone, and 2 per cent. dextrose (Von Ermengen). Heat at 60° C. for one hour. Incubated at 28° C. Filter, and inoculate a 250 gram guinea-pig with 1 c.c. of the filtrate. If the pig dies repeat the test administering botulinus antitoxin prior to the injection of the filtrate. Death of the first pig and survival of the second permits the conclusion that B. botulinus was present in the original material. Pure cultures can be obtained by anaërobic plate methods.

Bacillus Tuberculosis.—*Synonyms:* Tubercle bacillus; Bacillus of Koch.

Morphology.—Slender rods; in fresh cultures staining homogeneously; in older cultures presenting a segmented or irregularly stained appearance. They frequently occur in pairs of short rods and in closely adhering clumps and strands. When once stained with fuchsin or gentian-violet they are not decolorized by treatment with Gabbet's solution or with a solution of any of the mineral acids, followed by alcohol. In the sputum of pulmonary tuberculosis the bacillus sometimes occurs in filaments which branch. On this account the organism is considered by many to belong to the group of the streptothrices.

Stained by Gram's method. Not motile. Does not form spores.

There are two types pathogenic for humans: human and bovine.

Blood-serum.—After three or four weeks in the incubator the colonies appear as dry, cream-colored, granular, slightly elevated patches with irregular margins, 1 to 2 mm. in diameter. They may become confluent to form a dense, dry, granular mass with irregular surface and of a creamy-white color. The growth is very friable, but coherent, and may be picked up in clumps on the platinum wire. The first gener-
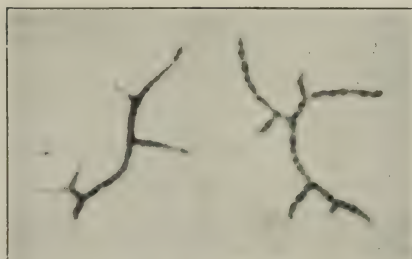
FIG. 97.—Branched tubercle bacilli from sputum; × 2000 (Wright and Brown).

ation from tissues is very slow in developing, but succeeding generations grow more rapidly, and may form a wrinkled, dry, cream-colored membranous layer on the surface of the medium.

Glycerin Agar Slant.—Growth similar to that on blood-serum, but not so vigorous. By continued inoculation of this medium through a number of generations, however, the organism may eventually grow luxuriantly upon it.

Glycerin Bouillon.—Growth on the surface in the form of floating patches or as a membrane similar in appearance to the growth on blood-serum. The growth sinks to the bottom from time to time. The glycerin-bouillon culture is best contained in Erlenmeyer flasks, filled to such a depth as to give a wide surface to the fluid and thus permit the access of plenty of oxygen to the growth.

Potato.—The growth is not remarkable.

Agar or bouillon not containing glycerin is not suitable for the cultivation of this bacillus.

Tuberculin.—Tuberculin is obtained from cultures of the tubercle bacillus. It is presumably an endotoxin and is derived from the bacilli themselves. Injected into a tuberculous subject in proper dosage it will produce a rise of temperature if introduced subcutaneously, conjunctival congestion if introduced into the conjunctival sac, and local erythema and induration, if introduced intradermally. The last method, rarely the first, is used for diagnosis in humans, and all methods, most commonly the first two, for diagnosis in animals. A positive diagnosis in guinea-pigs which have received tuberculous material can be made by intracutaneous injection two weeks or more after inoculation. In employing this method only those guinea-pigs should be used which have given a negative test prior to inoculation. In normal subjects the injection of corresponding amounts of tuberculin has no effect.

Pathogenesis.—The inoculation of guinea-pigs or rabbits by any method is followed by the development of general miliary tuberculosis. Guinea-pigs are most susceptible. These animals usually survive about two or three months, with marked emaciation. The lesions in the spleen and liver in the guinea-pig are characterized by extensive areas of necrosis not confined to the tubercular tissue, large parts of these organs being transformed into a firm yellow, opaque, friable material.

Isolation of the Bacillus Tuberculosis from Tuberculous Lesions. The tuberculous lesions in human tissues are not ordinarily favorable for the isolation of the bacillus, on account of the frequent presence of other bacteria in them and because of the small number of tubercle bacilli usually present in tissue otherwise suitable. The best method of procedure is to inoculate a guinea-pig subcutaneously in the abdominal wall with tuberculous material, and after four to six weeks, when the inguinal lymphatic glands have become enlarged, to kill the animal and make cultures on suitable media from tuberculous lymphatic glands. The object of killing the animal, rather than allowing it to die spontaneously, is to secure fresh tissue

and to avoid the chance of an invasion of the lesions by other bacteria.

For the cultivation of tubercle bacilli from tuberculous lesions cultures may be made to Dorset's egg-medium. Just before inoculating the medium three or four drops of sterile distilled water should be added to each tube to supply the moisture required for the satisfactory development of the tubercle bacillus.

After inoculation the tube should be placed in the incubator at 38° C. in an inclined position, so that the surface of the medium may keep moist. Colonies first become visible after seven or eight days in the incubator.

A number of tubes are to be inoculated, say three or four, from each of the two or three glands, a large quantity of material being spread upon the surface of each tube. Great care is to be exercised to avoid contamination with other bacteria in preparing these cultures. The culture-tubes used should contain freshly prepared moist medium, and immediately after inoculation should be sealed air-tight to prevent evaporation. This may conveniently be done by first cutting off the projecting portion of the cotton stopper and inserting a cork into the mouth of the tube in such a way as to push the cotton stopper before it.

In order to prevent the invasion of fungi from the cotton, the neck of the tube should be heated in the Bunsen flame until the cotton begins to brown before inserting the cork, which should also be charred in the Bunsen flame before insertion. The tubes may also be sealed with wax or paraffin or covered with small rubber caps.

S. A. Petroff's Method for the Cultivation of Tubercle Bacilli from Sputum and Feces.—Equal parts of sputum and 3 per cent. sodium hydrate solution are mixed and incubated for twenty to thirty minutes to liquefy the sputum. The alkali is then neutralized with normal hydrochloric acid, the mixture centrifuged, and cultures sown on a special medium which is prepared as follows:

An infusion of beef or veal is prepared by mixing 500 grams of finely ground meat with 500 c.c. of 15 per cent. glycerin

solution, and after twenty-four hours' standing in the ice-chest the mixture is passed through a sterile meat press and the fluid collected in a sterile beaker. This infusion is mixed with an equal volume of whole eggs and sufficient of a 1 per cent. alcoholic solution of gentian violet to make a dilution of 1 : 10,000. The mixture is tubed and inspissated in the form of "slants" at 85° C. and then heated for not more than one hour at 75° C. on each of the two following days. The shells of the eggs are sterilized by immersing in 70 per cent. alcohol for ten minutes or by pouring hot water over them. Before admixture with the infusion the whites and yolks of the eggs are beaten up in a sterile beaker and filtered through sterile gauze.

Feces are mixed with three volumes of water and filtered through gauze. The filtrate is then saturated with sodium chloride, allowed to stand for a half-hour, when the film at the surface containing the bacteria is transferred to a bottle, shaken up with equal parts of normal sodium hydrate solution, and left in the incubator for three hours, shaking up every half-hour. The mixture is then neutralized to litmus-paper with normal hydochloric acid, centrifuged, and cultures sown on the surface of the special medium.

The Antiformin Method for Obtaining Pure Cultures of the Tubercle Bacillus.—Antiformin is the patented name for a solution consisting of equal parts of liquor sodæ chlorinatæ and a 15 per cent. solution of caustic soda. It quickly dissolves mucus and the cells and fibers of animal tissues, and also has the remarkable property of destroying all bacteria except tubercle bacilli and other acid-fast bacilli.

Lawrason Brown and Daniel Smith have used the following procedure with great success in cultivating tubercle bacilli from the sputum in a series of cases:

Equal parts of a 30 per cent. aqueous solution of antiformin and sputum are thoroughly mixed in a sterile centrifuge tube and allowed to stand at room-temperature for one hour. The tube is then centrifugalized at high speed, the supernatant fluid decanted, and the sediment mixed with sterilized distilled

water. This is again centrifugalized, and the whole process is carried out three times. The sediment is then streaked over the surface of Dorset's egg medium and placed in the incubator.

Cultures from tuberculous tissue may also be obtained by a similar procedure, the tissue being ground up in a mortar with a 15 or 20 per cent. solution of antiformin, or frozen sections made of it and placed in the same solution. When the tissue has been dissolved, which occurs in the course of a few minutes, the solution is to be centrifugalized, the sediment washed, and cultures made from it as above described for sputum.

A. S. Griffith has found that it is not necessary to wash the bacilli free from antiformin in order to obtain cultures. Antiformin is mixed with the sputum in the proportion of 15 per cent. and a loopful of the mixture is spread over the surface of the egg medium in tubes, three tubes being sown at intervals of one or two minutes. Thereafter tubes are sown at intervals of five minutes if the sputum remains undissolved and mucinous. After twenty minutes or after complete solution the tubercle bacilli will not be viable.

Occurrence.—The organisms may be recovered from the sputum of pulmonary tuberculosis, from the feces in intestinal tuberculosis, from the urine in many cases of genito-urinary tuberculosis, from the spinal fluid in cases of tuberculous meningitis, and frequently from the discharges of lesions occurring on the skin, and in bones, joints, and lymph nodes. At autopsy they may be found in tubercles of the liver, brain, or spleen and in miliary tuberculosis are distributed everywhere throughout the body.

In children the organisms recovered from cases of cervical tuberculous adenitis, abdominal tuberculosis, and generalized tuberculosis of definite abdominal origin are more commonly of the bovine rather than of the human type. This type is recovered from cases of bovine tuberculosis and may be found in the milk from cows thus affected.

May occur on the surface of objects contaminated with the excreta of tuberculous individuals or in the dust of places inhabited by such individuals.

Diagnosis.—For clinical purposes the tubercle bacillus may be identified in smear preparations by means of the special methods of staining, which depend upon the fact that the tubercle bacillus, when once thoroughly stained with an aniline dye, does not give up its stain in the presence of acids, as nearly all other bacteria do. The tubercle bacillus may therefore be identified even among a mixture of other bacteria by this property, taken in connection with its morphology, in most of the routine work of the pathological laboratory. Practically, the only other bacilli with which it may be confounded are the bacillus of leprosy and the smegma bacillus, both of which, when stained, resist the decolorizing action of acid. It may be differentiated from the smegma bacillus by the fact that it is not decolorized by alcohol (95 per cent.) after the usual treatment with acid, while the smegma bacillus is decolorized under these circumstances. A modification (Pappenheim) consists in immersing the slide without washing after staining in the usual way in carbol-fuchsin in a 1 per cent. alcoholic solution of rosolic acid saturated with methylene blue. The tubercle bacilli appear red and the smegma bacilli blue.

As a rule, the differential test need only be applied in the examination of urine and the material derived from about the external genitalia, especially in the case of females.

The differentiation from the bacillus of leprosy by certain quantitative differences in staining reactions has been attempted, but it is very unsatisfactory, and it is doubtful if there is as yet any reliable method of distinguishing between these two organisms, considered by themselves.

Differentiation of Types.—In order to trace the source of tuberculous infection the laboratory is sometimes asked to differentiate between the two main types of the bacillus— human and bovine. One which morphologically has a tendency to be plump, which produces a final slightly alkaline reaction in glycerine broth, which grows slowly and sparsely on artificial media and is not materially aided by the presence of glycerine, and which in small amounts consistently causes death in rabbits within two months is presumably of the bovine type.

The avian type grows more readily and rapidly and at a higher temperature (41°–45° C.) than either the human or bovine. In glycerine broth it grows not only on the surface but also within the media. It is extremely pathogenic for rabbits and practically non-pathogenic for guinea-pigs.

Examination of Sputum for Tubercle Bacilli.—The morning sputum should be taken for examination. Select one of the

FIG. 98.—Tubercle bacilli in sputum (carbol-fuchsin and methylene-blue) (Vierordt).

dense, grayish-white particles, and with the aid of small-pointed forceps or the platinum wire rub it over the surface of a cover-glass or slide, breaking it up as much as possible. The material should be spread in a very thin layer. The preparation is next to be fixed in the ordinary way, and is then to be treated as follows:

1. Stain in carbol-fuchsin solution, steaming for one to four minutes over a Bunsen flame or water bath, with the staining solution thoroughly covering all the surface of the cover-glass

or slide.   None of the surface should be allowed to become dry by evaporation, as this causes a precipitate to form, but more of the staining fluid should be added from time to time to keep it completely covered as evaporation occurs.   The object of the heating is thoroughly to impregnate the bacilli with the dye.

2. Wash in water.

3. Decolorize in acid alcohol until the red color disappears. Do not allow the acid to act on the preparation longer than a few seconds.   The solution should also be applied to the uncharged side of the cover-glass or slide to remove any dried stain which may have collected thereon.

4. Wash thoroughly in water.

5. Wash in 95 per cent. alcohol for thirty seconds.

6. Wash in water.

7. Stain in Löffler's methylene-blue solution for thirty seconds.

8. Wash in water, and dry.

The tubercle bacilli are stained red and the nuclei of cells and other bacteria are stained blue.

Antiformin Method.—The finding of tubercle bacilli in sputum is greatly facilitated by making smear preparations, as above described, from the sediment obtained by the antiformin method described on page 390.

In order to destroy any extraneous tubercle bacilli or other acid-fast bacilli which may be in the centrifuge tube, just before use the interior of the tube is to be thoroughly exposed to the action of concentrated sulphuric acid saturated with potassium bichromate, after which the tube is to be thoroughly washed with distilled water.   Sputum cups, or other receptacles of crockery or of glass used to collect the sputum, should be treated in the same way.

It is of the greatest importance to be sure that the distilled water used does not contain acid-fast bacilli which sometimes develop in it.

As antiformin does not kill tubercle bacilli, the centrifuge tube should be sterilized after use.

In a very few cases of gangrene of the lung bacilli like smegma bacilli have been found in the sputum.   These may be mistaken for tubercle bacilli (*vide ante*).

Tubercle Bacilli in Urine.—The sediment of the urine should be examined.   This may be rapidly thrown down by the

centrifuge. If it is abundant, the urine should be first centrifuged at low speed, and then transferred to another tube and centrifuged for an hour at high speed. It is important that the tubes be mechanically clean and free from any bacilli from previous specimens of urine. With the sediment smear preparations are to be made and stained as described for sputum. Especial care should be taken to wash thoroughly in alcohol after the decolorization with acid, in order to decolorize any smegma bacilli that may be present. (See remarks on Diagnosis, page 392.) Because smegma bacilli may be mistaken for tubercle bacilli and because the tubercle bacilli may be so few as to escape observation, the inoculation of a guinea-pig with the sediment is the better test for the presence of tubercle bacilli in the urine. If, however, the urine be obtained by catheter from a ureter, the first objection is practically eliminated.

For inoculation the urine should be collected in sterilized vessels and immediately centrifugalized in sterilized tubes. The sediment is then to be injected subcutaneously into a guinea-pig with a sterilized syringe.

Tubercle Bacilli in Spinal Fluid.—In cases of tuberculous meningitis the spinal fluid is usually clear. On standing a fine white clot forms and it is often possible to find the organisms tangled up in this clot. After the clot has formed the contents of the tube should be poured into a sterile Petri dish. On the surface float a piece of rice paper such as is used for rolling cigarettes. The clot will adhere to the surface of the paper. Carefully lift off the paper and place it clot side down on a clean slide. Blot firmly. On removing the paper the clot will be found well spread out and firmly adherent to the slide. Fix and stain in the usual manner. Any attempt to remove the clot by means of the platinum loop will result disastrously, as the fine fibrin shreads cling tenaciously to the wire.

For inoculating animals the sediment obtained from centrifuging as large an amount of spinal fluid as can readily be obtained is injected into a guinea-pig in the usual manner.

Tubercle Bacilli in Tissues, Pus, and Feces.—The bacilli may be demonstrated in the following ways:

1. By the staining of the bacilli in sections of tissue by the special methods described on pages 397 and 398. Frozen sections prepared by the method elsewhere described may be employed.

2. By making smear preparations and staining as described for sputum. These preparations may be made directly from the material; but if the bacilli are few, as is usually the case, they should be made from the sediment obtained by the anti-formin method described on page 390. This method is also applicable to fixed and hardened tissue, even if it has been imbedded in paraffin. The paraffin should be thoroughly removed from the sections by means of xylol, followed by absolute alcohol, before placing them in the solution of antiformin.

The precautions against error from the presence of extraneous tubercle bacilli, or other acid-fast bacilli, are to be taken which are described in connection with the application of this method to the examination of the sputum.

3. By the inoculation of guinea-pigs with the material or sediment obtained by the antiformin method. The inoculation is best made subcutaneously in the abdominal wall, either with a hypodermic syringe, if the material be fluid, or if it is in the form of tissue, by inserting a small piece beneath the skin. Material obtained on a swab may also be used for inoculation by introducing the infected swab beneath the skin and moving it back and forth a few times. If tubercle bacilli are present in the material, the animal will show enlargement of the inguinal lymph nodes in about three weeks, and will usually die of miliary tuberculosis in the course of six to ten weeks. If necessary, the nodes in the inguinal region may be examined histologically after three weeks for the presence of tuberculous lesions, or examined by smear preparations for tubercle bacilli.

To Stain Tubercle Bacilli in Paraffin Sections.—The important point about staining *tubercle bacilli* is to stain them deeply

enough in the beginning; then there is little danger of their fading in the subsequent steps of contrast staining. It is probable that carbol-fuchsin, used hot, is the most powerful stain we have for this purpose. If the solution is steamed, generally on the slide, one to five minutes are probably sufficient for all purposes. Tubercle bacilli stain well, not only after alcohol, but also after most of the other fixing reagents, such as corrosive sublimate, Zenker's fluid, Fleming's solution, etc.

Ehrlich's Method.—1. Stain paraffin sections in aniline-fuchsin or methyl-violet for half an hour to twenty-four hours, or for one to five minutes if solution is heated to steaming.

2. Wash in water.

3. Decolorize in 20 per cent. nitric acid one-half to one minute.

4. Wash in 70 per cent. alcohol until no more color is given off.

5. Contrast-stain in a saturated aqueous solution of methylene-blue or of Bismarck brown one to two minutes.

6. Wash in water.

7. Dehydrate in absolute alcohol.

8. Xylol, xylol balsam.

Ziehl-Neelson-Gabbet Method.—1. Stain paraffin sections in carbol-fuchsin solution, warming the solution so that it steams one to three minutes.

2. Wash in water.

3. Decolorize and stain for contrast in sulphuric-acid-methylene-blue solution one minute (see page 75).

4. Wash in water.

5. Absolute alcohol.

6. Xylol.

7. Xylol balsam.

This method is not suited to celloidin sections, because the celloidin retains too deep a blue stain.

Kühne's Method.—1. Stain paraffin sections lightly in alum-hematoxylin.

2. Wash in water.

3. Stain in carbol-fuchsin one to five minutes if warmed, longer if cold.

4. Wash in water.

5. Aniline hydrochlorate, 2 per cent. aqueous solution, fifteen seconds.

6. Wash in water.

7. Absolute alcohol.

8. Xylol.

9. Xylol balsam.

To Stain Tubercle Bacilli in Celloidin Sections.

1. Stain rather lightly in alum-hematoxylin.
2. Wash in water.
3. Dehydrate in 95 per cent. alcohol.
4. Attach sections to slide by the ether-vapor method.
5. Carbol-fuchsin two to five minutes steaming.
6. Water.
7. Orth's discharging fluid (acid alcohol) one-half to one minute.
8. Wash thoroughly in several changes of water to remove acid completely and to bring back blue color to nuclei.
9. Alcohol 95 per cent. until fuchsin is entirely discharged.
10. Aniline followed by xylol; or blot and treat with xylol.
11. Xylol balsam.

The advantages of this method are—that the celloidin is colorless; the nuclei are stained blue; the rest of the tissue is colorless; the tubercle bacilli stand out in sharp contrast. It is sometimes an advantage to bring out the cell-protoplasm and the intercellular substance by staining the sections, after decolorization in alcohol, in an aqueous solution of orange G or methyl-orange for a few seconds.

Bacillus Lepræ (Leprosy Bacillus).—This bacillus resembles closely the tubercle bacillus in morphology and staining reactions. It is somewhat less resistant than that bacillus to decolorization by acids.

Cultures.—There is no satisfactory evidence that this organism has ever been obtained in pure culture.

Occurrence.—The bacillus often grows in enormous numbers in the lesions, chiefly in the cytoplasm of endothelial leucocytes, where they often lie parallel to one another in bundles. They may also be found in nerves and in nerve-cells.

Diagnosis.—Diagnosis can often be made by examining smear preparations of scrapings from the nasal cavity. These should be stained as for tubercle bacilli bearing in mind that the organisms are much more readily decolorized than tubercle bacilli. Light swabbing of the nasal cavity will not suffice for the organisms are contained in the epithelial cells and for a positive diagnosis these cells must be dislodged.

To Stain the Bacillus of Leprosy in Sections.—The bacillus of leprosy stains more easily than the tubercle bacillus. Simple

aqueous solutions of the aniline dyes are sufficient. The Gram-Weigert stain gives a brilliant picture. The same methods can be employed as for tubercle bacilli if a differential stain is desired. A method recommended by Flexner will be found very useful.

1. Stain in alum-hematoxylin so as to get a sharp nuclear stain.
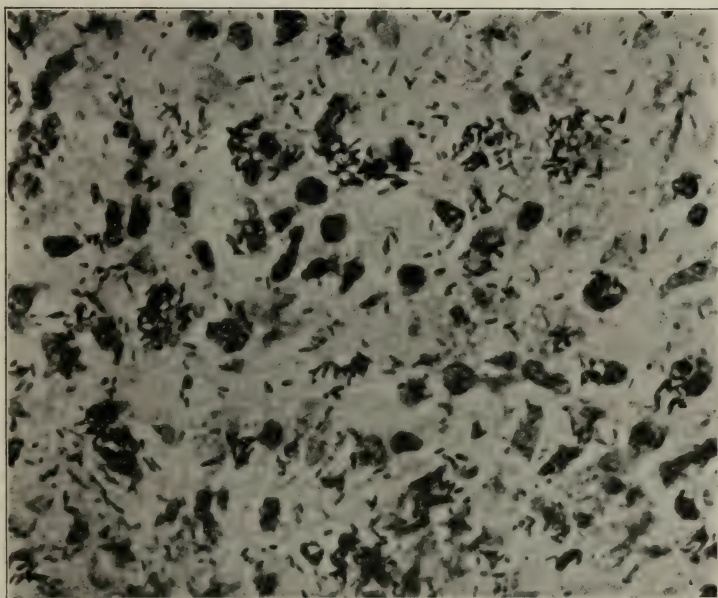
FIG. 99.—Bacillus of leprosy: section through a cutaneous nodule, showing the bacilli in the tissue; × 750 (Wright and Brown).

2. Wash in water.

3. Carbol-fuchsin two to five minutes steaming, or thirty to sixty minutes cold.

4. Water.

5. Treat on the slide with iodin solution one-half to one minute.

6. Water.

7. Blot; clear and differentiate in aniline oil.

8. Xylol; balsam.

Baumgarten gives the following differential stain for leprosy bacilli:

1. Stain six to seven minutes in a dilute solution of fuchsin (5 drops of a concentrated alcoholic solution to a watch-glass of water).

2. Discharge one-quarter minute in nitric acid alcohol (nitric acid 1, alcohol 10).

3. Wash in water.

4. Contrast-stain in a saturated aqueous solution of methylene-blue.

5. Alcohol.

6. Xylol.

7. Balsam.

While leprosy bacilli stain readily by this method, tubercle bacilli will not stain in so short a time.

Spirillum Choleræ Asiaticæ (Comma Bacillus).—*Morphology* (Figs. 100, 101).—In fresh cultures the organism
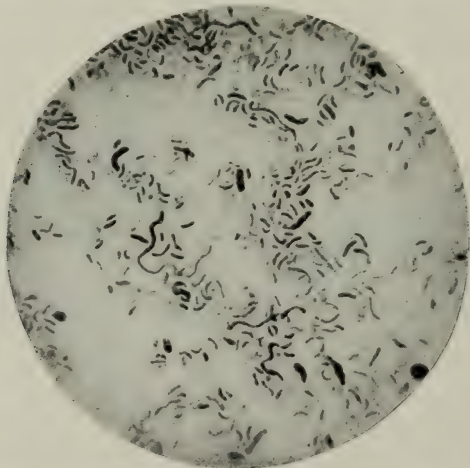
Fig. 100.—Spirillum of Asiatic cholera, from a bouillon culture three weeks old, showing long and degenerate forms; × 1000 (Fränkel and Pfeiffer).

appears usually as a slightly curved rod somewhat shorter than the tubercle bacillus, but much thicker. The curving of the rod varies, being very marked in some individuals and absent in others. Sometimes two rods are joined end to end with their convexity pointing in opposite directions, or moderately long, undulating threads may be found. It

seems probable that the curved rods represent the segments of a spirillum, and hence the name of the organism.

In cultures some days old degenerated and atypical forms are found (involution forms). The organism is motile, and a single flagellum is attached to the end of the rod.
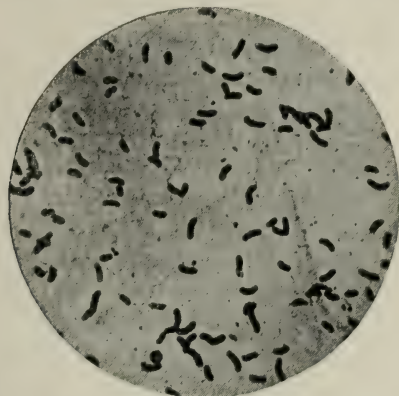
FIG. 101.—Spirillum of Asiatic cholera, showing the flagella; × 1000 (Günther

It is not stained by Gram's method; motile.

Colonies on Gelatin Plates (Fig. 102).—After twenty-four to forty-eight hours at a temperature of 20° to 22° C. the largest colonies will appear as masses of indefinite granular
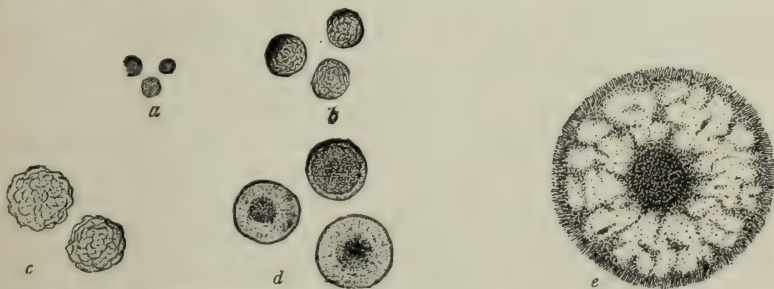
FIG. 102.—Developmental stages of colonies of the spirillum of Asiatic cholera at 20° to 22° C. on gelatin; × about 75 diameters (Abbott): *a*, after sixteen to eighteen hours; *b*, after twenty-four to twenty-six hours; *c*, after thirty-eight to forty hours; *d*, after forty-eight to fifty hours; *e*, after sixty-four to seventy hours.

material lying in circular areas of liquefied gelatin in which granular shreads are scattered. Within the next twenty-four hours the areas of liquefaction increase, and the colonies

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appear under the low power "as a dense granular mass sur-rounded by an area of liquefaction through which can be seen granular prolongations of the colony, usually extending irregu-larly between the periphery and the central mass" (Abbott), while the margin of the liquefied area is marked by delicate radiating filaments closely packed together.

Agar.—Grayish, opalescent surface colonies.

Gelatin Stab.—Growth all along the line of inoculation with liquefaction at the surface in funnel form after forty-eight hours. The liquefaction proceeds in such a manner that the liquefied area has a smaller diameter at the surface than immediately beneath, and, owing to the fact that the liquefied gelatin does not fill the cavity, a space is left between the surface of the medium and the surface of the liquefied gelatin so that the appearance of an air-bubble is produced. Along the deeper portions of the line of inoculation the lique-faction is slow.

Bouillon.—Diffusely clouded. A thin pellicle forms on the surface after a time.

Litmus-milk.—Turned red and coagulated.

Indol-production.—In cultures in Dunham's pepton solu-tion or in the pepton solution of Koch (2 per cent. pepton and 1 per cent. sodium chlorid) a rose-color is produced by the addition of sulphuric acid alone (concentrated c. p. acid is to be employed, as in the test for indol-production by Bacillus coli communis). The production of the rose-color without the addition of the sodium nitrite shows that nitrites as well as indol are formed by the growth of the organism in the pepton solution. The reaction can be obtained in cultures which have been but eight hours in the incubator.

Potato.—Thin, dry, grayish-white growth which does not spread over the surface.

Pathogenesis.—Individual strains varying greatly in virulence. Subcutaneous injections into guinea-pigs and rabbits are rarely ever fatal. Intraperitoneal injections of any considerable numbers usually result in death. Dogs, cats, and mice are relatively non-susceptible.

Occurrence.—In the alvine dejections and in the intestinal contents of cholera patients (Fig. 103). It apparently only rarely invades the circulating blood. Its presence in the vomitus may sometimes be shown. It has been found in the water-supplies during epidemics.

The cholera spirillum is the representative of a large group of spirilla, many of which may be found in river waters.
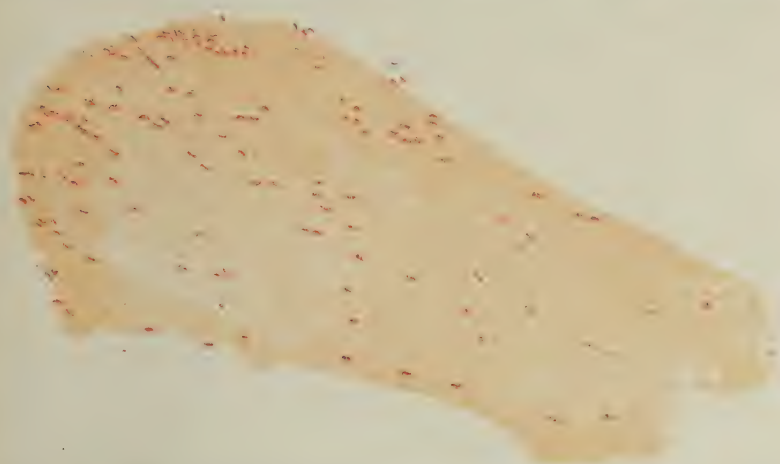
FIG. 103.—Smear preparation of a mucous floccule in Asiatic cholera; × 650 (Vierordt).

According to Abbott and Bergey, the only trustworthy method of distinguishing some of these from the true cholera spirillum is their failure to manifest agglutination with the serum of an animal immunized against the true cholera spirillum.

Bacteriological Diagnosis.[1]—Cultures should be made from the feces, or contents of the lower end of the ileum, in a special fluid medium and on agar plates. Mucous flakes, if possible, should be taken for inoculation. The special fluid medium favors the growth of spirilla and is prepared as follows:

Pepton (Chapoteau or Witte)....................	10.0
Salt...	10.0
Potassium nitrate...............................	.1
Sodium carbonate..............................	.2
Distilled water..................................	1000.0

[1] McLaughlin, A. J.   Reprint from *Public Health Reports*, No. 53.   Public Health and Marine Hospital Service of the United States.

The agar plates are made up with 15 c.c. each of 3 per cent. agar, which has been made alkaline by the addition of 3 c.c. of a 10 per cent. solution of caustic soda to each 100 c.c. of the medium after it has been made neutral to litmus.   The plates are inoculated in sets of three after the agar has solidified by rubbing one loopful over the surface of the agar in one plate with a platinum loop or a bent glass rod, and then streaking the surfaces of the other plates successively with the same loop or rod.   The surfaces of the solidified agar must be dried before inoculation by placing the plates for five minutes in a warming oven at 60° C., or in the incubator at 37° C., for one hour, with the covers removed and the agar surface downward. The tubes of the fluid medium should be inoculated with one loopful of the material and the flasks with 1 c.c.

After inoculation all cultures are placed in the incubator at 37° C.

The uppermost layers of the fluid cultures should be examined microscopically after three, six, twelve, and twenty-four hours without disturbing the fluid more than is necessary.   If spirilla are found agreeing in morphology, motility, and staining reactions with the cholera spirillum, agar plates are to be inoculated from this uppermost layer in the same manner as from the original material and incubated at 37° C.

The colonies on the agar plates develop within eighteen hours, and appear as pale, semitransparent discs, which show by transmitted light an opalescent or iridescent quality.   Suspicious colonies are to be tested as follows: On a clean glass slide are placed at three separate points single drops of a 1:200 dilution in physiological salt solution of an agglutinating cholera serum.   These drops are numbered on the slide 1, 2, and 3.   With them are then mixed portions of suspicious colonies by means of a straight platinum wire.   If the diffuse cloudiness of the drop of fluid changes within a few minutes to a clear fluid with flocculi in suspension, and the macroscopical and microscopical appearances of agglutination are produced, the colony is probably that of the cholera spirillum.   It may be necessary to test in this way numerous colonies.   From colonies thus giving a positive agglutination reaction agar slants are inoculated and incubated for eighteen hours, when emulsions of the spirilla for more delicate agglutination tests are prepared by pouring into each tube 5 to 8 c.cm. of sterile physiological salt solution and shaking the tubes.   Suspicious colonies not showing agglutination reactions should also be planted on agar slants and the growth tested again, because freshly isolated cholera spirilla do not always respond to the test.

The more delicate agglutination tests are carried out as follows: In each of a number of small test-tubes of 2 c.cm. capacity is placed ½ c.c. of dilutions in salt solution of agglutinating serum varying from 1 : 10 to 1 : 4000, or up to the limit of the agglutinating power of the serum.   To each tube is then added ½ c.c. of the emulsion of the suspected spirilla. These manipulations are carried out with a pipette, to which is attached a

rubber bulb for suction and expulsion. The highest dilutions at which agglutination appears in the tubes is noted after they have been in the incubator at 37° C. for one hour, and again after an additional two hours at room temperature. If the spirilla are true cholera spirilla, they will be agglutinated at or near the maximum dilution at which the specific serum agglutinates the true cholera microorganism. Of course the spirilla should also be shown to manifest the other characteristics described above before a positive diagnosis is made.

Dieudonné's Blood-agar Medium.—This has an inhibiting effect on the growth of other micro-organisms than spirilla, and may be employed in the same manner as the agar medium described above. It is prepared as follows:

Defibrinated ox blood	30
Normal solution of caustic potash	30
Nutrient agar (3 per cent.)	140

Add the caustic potash solution to the ox blood and add the melted agar. Sterilize for one hour at 100° C., and use about 15 to 20 c.c. for each plate.

The Micro=organism of Actinomycosis.—The proper name of this micro-organism is "Actinomyces bovis." It belongs to the group of filamentous branching micro-organisms which are regarded as occupying an intermediate position between the bacteria, on one hand, and the moulds or hyphomycetes on the other.

The organism appears in the pus from subacute or chronic suppurative lesions of the disease, actinomycosis, as grayish or yellowish granules, usually less than 1 mm. in diameter. Sometimes these granules are aggregated in groups of two or three, and thus appear as lobulated larger granules. They are friable, and when gently crushed beneath a cover-glass and observed under the microscope, they are seen to have been broken up into hyaline rounded masses, at the margins of which, on close inspection, fine radial striations or filaments or hyaline club-shaped bodies, all closely set together, may be seen (Figs. 104, 105). The club-shaped bodies are variable in .size, and are composed of a hyaline, refringent substance. The appearance of radial striation in the granule, when observed with the microscope, due to the presence and radial arrangement of these hyaline bodies, gave rise to the name "ray-fungus"

for this parasite.   Not all of the granules have these "clubs."
In the granules obtained from the lesions in man they are much
less frequently observed than in those obtained from the lesions
in cattle.
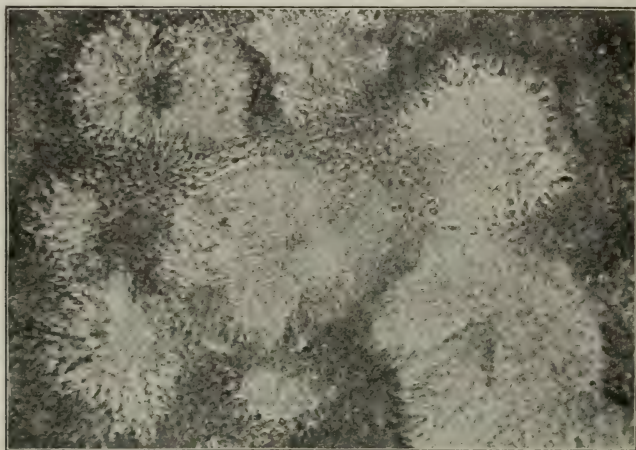
FIG. 104.—Actinomyces granule crushed beneath a cover-glass, showing radial
triations in the hyaline masses.   Preparation not stained; low magnifyings
power (Wright and Brown).

If a cover-glass or slide preparation be made by breaking up
one of the granules and staining with Gram's method, there will
usually be found, upon examination with an oil-immersion
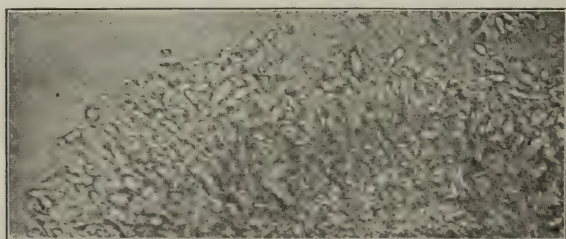
FIG. 105.—A portion of an actinomyces granule crushed beneath a cover-
glass, showing the "clubs."   The preparation not stained; moderately high mag-
nifying power (Wright and Brown).

lens, isolated and matted filaments, many of which may be
seen to branch, in addition to longer and shorter fragments
of filaments and fine detritus of the same (Fig. 106).   The
filaments are usually ·more or less wavy in their course, and
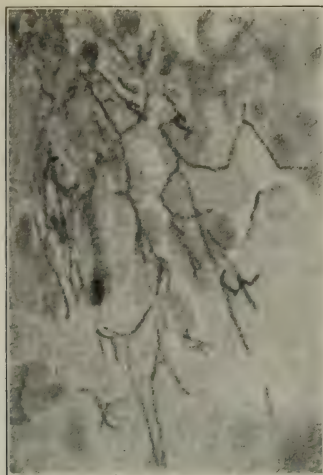
FIG. 106.—Branching actinomyces filaments in a smear preparation made from an actinomyces granule stained by Gram's method; × 1000 (Wright and Brown.)
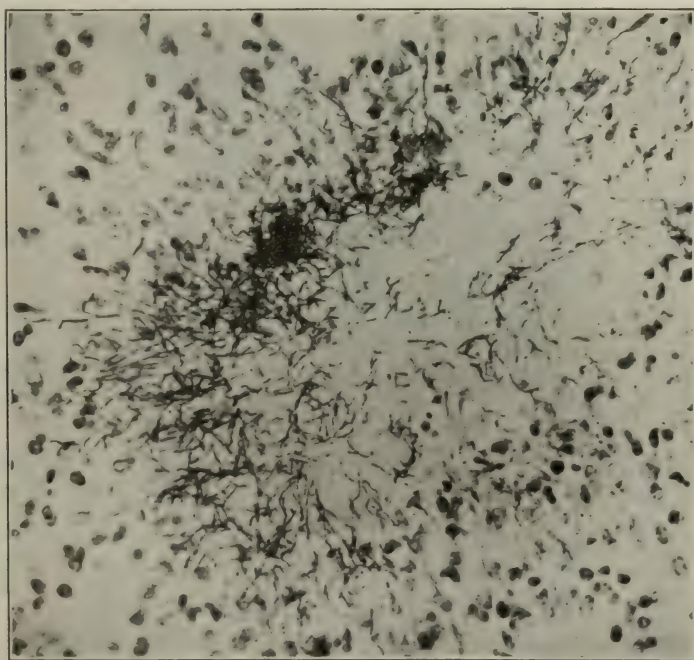
FIG. 107.—Colony or granule of actinomyces in a section through a lesion, showing the Gram-stained filaments and hyaline material and also the pus-cells surrounding the colony (Wright and Brown).

are, as a rule, slightly thicker than the tubercle bacillus. Some of the filaments will be found to stain homogeneously; others do not stain so deeply, and show numerous deeply staining points in their substance. If clubs are present in the granule, they also may be found scattered throughout the preparation.

In sections of the tissues stained by Gram's method two chief forms of granules are found. In one of these forms the granule is seen to consist of filaments embedded in a hyaline substance, and usually arranged at the periphery in an indefinite radiate manner (Fig. 107). At the margin of the granule the
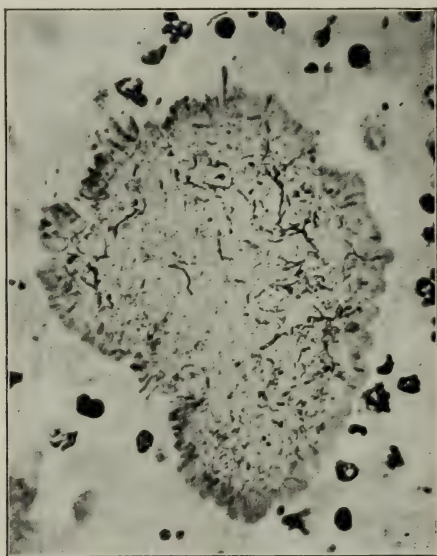
FIG. 108.—Colony or granule of actinomyces in a section through a lesion, showing the peripheral arrangement of the "clubs." In several instances the central stained filaments in the "clubs" are seen; × 750 (Wright and Brown).

filaments are usually much more numerous than in the central portions, where the hyaline material predominates. This hyaline material apparently consists of degenerate or dead filaments or their remains. The other form of granule seen in sections is distinguished by possessing at its margin a row of closely set radiating club-shaped bodies composed of hyaline substance which does not stain by Gram's method (Fig. 108). These are the "clubs" previously mentioned, and they may

occupy more or less the circumference of the granule. In certain instances a Gram-staining filament may be seen in the central portion of a club. The main mass of this form of granule is not essentially different from that of the first-mentioned form. The characteristics of both forms of granule may be found in some granules.

The club-shaped bodies are to be regarded as products of degeneration of the marginal filaments.

In some cases isolated or small groups of filaments may be found scattered among the pus-cells in the lesions.

Diagnosis.—The finding of the granules in suspected pus may be facilitated by spreading the pus on a slide.

The identification of the organism is made certain only when the granules have been found to present the appearances described above after crushing under a cover-glass, and after smear preparations made from them and stained by Gram's method show the branching filaments.

Cultures.—Actinomyces bovis is essentially an anaërobe and it does not grow at room-temperature. A good growth in cultures is obtained only in the depths of solid culture-media and in bouillon. Growth is obtained on the surface of solid culture-media only when large numbers of the micro-organisms are planted upon the culture-media. These sur-face growths are white, elevated, more or less nodular, and have irregular margins.

Sugar Agar.—In "stab" cultures and in cultures by the method of "Liborius" (see page 255), growth occurs only below a depth of about 1 cm. from the surface. The colonies continue to develop during some days in the incubator. The larger colonies are spherical, whitish, and may attain a diameter of 1 mm. or more. The smaller colonies, under the microscope, are seen to consist of a dense, interlacing felt-work of frequently branching filaments, which at the periphery are disposed in a more or less radiating manner. The microscopical colonies may be conveniently studied in thin slices cut out of the agar or in frozen sections of the agar fixed in formalin and stained by the Gram-Weigert method.

Bouillon.—Growth occurs in the form of solid, whitish masses in the bottom of the tube; there is never growth on the surface. When first isolated from the lesions the growth usually appears in the form of small, nodular, irregular, spherical, whitish structures, often adherent to one another, and forming mulberry-like masses, but under continued cultivation most of the strains of the micro-organism finally grow in the form of flaky, friable, amorphous masses, which in some instances, after some days in the incubator, become transformed into a stringy, viscid material. With most strains of the micro-organisms the bouillon remains clear. There is a good growth in bouillon, without any anaërobic precautions, apparently because the dense masses in which the micro-organism grows furnish sufficient anaërobic conditions within themselves.

Potato.—No growth.

The production of "clubs" outside of the body may be obtained by placing some of the nodular growth from a bouillon-culture in sterile serum or pleuritic fluid and keeping it in the incubator for a few days. The filaments of the micro-organism in immediate contact with the fluid become invested with the hyaline eosin-staining sheath, and the filament thus enclosed may no longer stain by the Gram-Weigert method. In this way structures are produced which are identical in every respect with the "clubs" developed from the filaments in the lesions. (See Figs. 105, 108, 109, 111.)

Pathogenesis.—Intraperitoneal inoculation of guinea-pigs with suspensions of the growth in bouillon-cultures produces, after three or more weeks, with some strains of the micro-organism, granulomatous nodules in the abdominal cavity, varying in size up to 1 cm. in diameter. These nodules consist of granulation and connective tissue, enclosing small abscesses in which are found the characteristic "club-bearing" colonies or granules. Different strains of the micro-organism vary in virulence and some produce no lesions.

Method of Isolation.—The granules, preferably obtained from closed lesions, are first thoroughly washed in sterile water or bouillon and then crushed and disintegrated between

two sterile glass slides. It is well to examine microscopically the disintegrated material to see if filamentous masses are
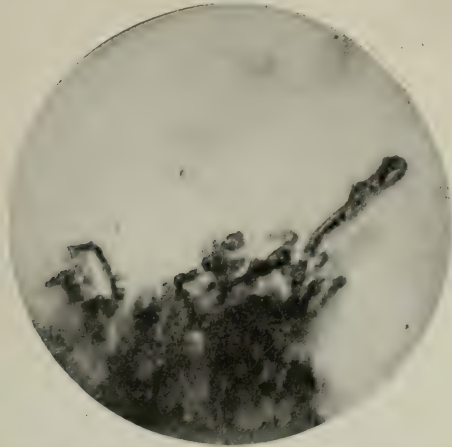
FIG. 109.—Showing "club" formation about the filaments of Actinomyces bovis, after exposure to the action of serous fluid outside of the animal body (Wright and Brawn).

present, because in some instances, through degenerative changes, the filaments which represent the living elements
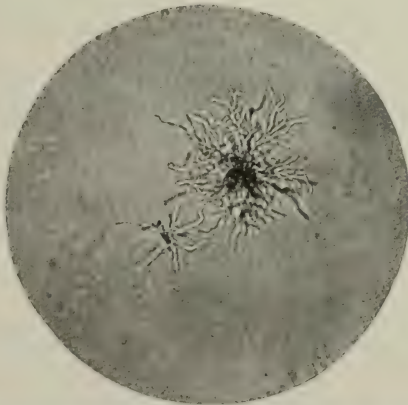
FIG. 110.—Small colonies of Actinomyces bovis in the depths of an agar culture.

of the granules have died out or disappeared from the granules. If no filaments are present, or if they are few in number, it is not

advisable to proceed further. If, however, filaments and filamentous masses are found, then the disintegrated products of the granules are to be transferred by means of the platinum loop to melted 1 per cent. dextrose-agar, contained in test-tubes filled to a depth of about 7 or 8 cm., which have been cooled to about 40° C. The material is to be thoroughly distributed throughout the melted agar by means of the loop, and the tube then placed in the incubator. At the same time a number of granules, after thorough washing in sterile water or bouillon, should be placed in sterile test-tubes, plugged with cotton, and kept at room temperature in the dark.

The sugar-agar tubes, inoculated as above described, should be examined from day to day for the presence of the characteristic colonies in the depths of the agar. If very many colonies of contaminating bacteria develop in the tubes, it will probably be very difficult or impossible to isolate the specific micro-organism. If there are a few or no contaminating colonies, then the colonies of the specific micro-organism should be expected to develop in the course of two or three days to a week. If a good number of living filaments of the micro-organism have been distributed throughout the agar, the specific colonies that develop will be very numerous in the depths of the agar, especially throughout a shallow zone situated about 1 cm. below the surface of the agar-agar.

When the presence of the characteristic colonies has been determined, slices or pieces of the agar, containing colonies, are to be cut out of the tube by means of a stiff platinum wire with a flattened and bent extremity. A piece of the agar is to be placed on a clean slide and covered with a clean cover-glass. It is to be examined under a low power of the microscope, and an isolated colony selected for transplantation. By obvious manipulations, under continuous control of microscopic observation, the selected colony, together with a small amount of the surrounding agar, is to be cut out, care being taken that no other colony is present. The small piece of agar thus cut out should not have a greatest dimension of more than 2 mm. The piece of agar is then transferred from the slide by

means of a platinum loop to a tube of sterile bouillon, where it is thoroughly shaken up in order to free it from any adherent bacteria.   If there be reason to believe that the small piece of agar has been very much contaminated with bacteria, it should be washed in a second tube of bouillon, then the piece of agar is to be transferred by means of the platinum loop to a tube of melted sugar-agar cooled to 40° C.   It should be deeply immersed in the agar and the tube placed in the incubator. If the colony thus transferred to the agar is capable of growth,
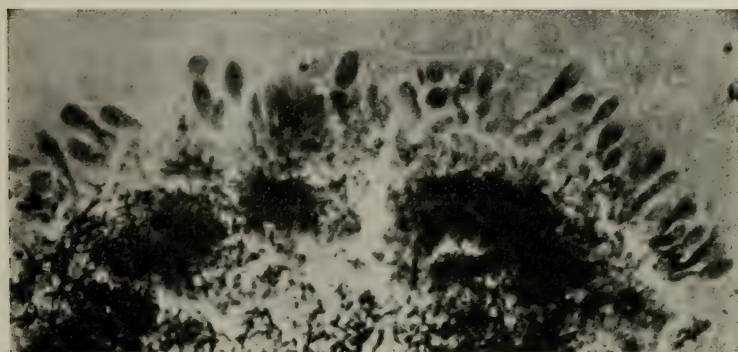
FIG. 111.—Portion of a colony of Actinomyces bovis in a section from a lesion in a guinea-pig produced by intraperitoneal inoculation.   The radiating "clubs" at the periphery, some with central filaments, are shown, as well as the felt-work of interlacing branching filaments in the central portions (Wright and Brown).

in the course of some days it will have formed a good-sized colony from which transplants in various culture-media may be made.

In the manner described several small pieces of agar containing single isolated colonies should be placed in sugar-agar tubes, because the chances are that some of the colonies will not grow, and contaminations with bacteria may occur.

If the number of contaminating colonies is so great in the original agar-cultures from the granules that it is found impossible or very difficult to obtain specific colonies free from other micro-organisms, then it is probably not worth while to expend much labor with the task of isolation from these original agar-tubes, but it is much better to wait until the

granules placed on the sides of sterile test-tubes have dried thereon for two or three weeks, and then proceed with these granules as just described for the fresh granules. The drying of the granules for this length of time will probably suffice to kill off most of the contaminating bacteria and enable isolated colonies of the specific micro-organism to be obtained in the agar suspension-cultures.

To Stain the Actinomyces in Sections.—In staining the actinomyces it is important to stain not only the filaments and other forms of the organism but also the hyaline swollen sheaths which surround the ends of the filaments. Eosin followed by methylene-blue sometimes gives good results. Good preparations can also be obtained by staining in alum-hematoxylin, followed by a strong solution of eosin; place the sections for five to thirty seconds in acid alcohol, and then wash thoroughly in water before dehydrating in alcohol. It is believed that the two following methods will give better results than can be obtained by any of the methods previously published for this purpose. The first is, perhaps, the better and surer, although the clubs are sometimes brought out more intensely by the second method.

Formaldehyde and alcohol fixation are preferable to Zenker's fluid for the study of this micro-organism, but not for the study of the lesions produced by it.

Mallory's Stains.—Method No. 1.—1. Stain sections deeply in a saturated aqueous solution of eosin for at least ten minutes.

2. Wash off in water.

3. Stain in aniline-methyl-violet two to five minutes.

4. Wash off with normal salt solution.

5. Iodin solution (1:2:100) one minute.

6. Water. Blot with filter-paper.

7. Aniline oil until section is clear.

8. Xylol, several changes.

9. Xylol balsam.

A light preliminary stain with alum-hematoxylin will often be found useful to bring the nuclei out sharply.

Method No. 2.—1. Stain lightly in alum-hematoxylin three to five minutes.

2. Wash in water.
3. Dehydrate in 95 per cent. alcohol.
4. Fasten section to slide with ether-vapor.
5. Aniline-methyl-violet five to twenty minutes.
6. Wash off with water.
7. Dry with filter-paper.
8. Aniline saturated with fuchsin one to three minutes.
9. Wash out the fuchsin with pure aniline until the clubs are sharply differentiated: watch the process under the low power of the microscope.
10. Xylol, several changes.
11. Xylol balsam.

The polymorphous bacterium is stained blue, the swollen membrane (the club), light to dark pink. By these methods it is possible to demonstrate in sections containing young colonies the ends of the threads stained blue surrounded by the swollen cell-membrane stained pink.

Sporotrichum Schenckii.—This is a fungus or hypomycete which is believed to be the infectious agent in sporotrichosis, a disease characterized by the formation of gumma-like nodes, abscesses, and ulcers chiefly involving the skin and subcutaneous tissue.

Morphology.—In the lesions of the spontaneous disease the fungus elements are difficult or impossible to distinguish from cellular detritus, apparently because they exist therein in certain small spore-like forms.

In culture the fungus appears in the form of a mycelium composed of branching septate filaments with abundant formation of spores. The filaments vary somewhat in thickness, their average being about 2 microns, have refringent walls, refractive granules in their interior, and transverse septa at fairly regular intervals. The spores grow singly along the sides of the filaments and in clusters of from 3 to 6 or more at the ends of filaments, which may be slightly expanded. They are ovate or spiculate bodies, 3 to 5 microns in their longest diameter, having a granular interior and a double contour. They are attached to the filaments by delicate pedicles, which are easily broken. With basic aniline dyes and with Gram's method of staining they stain generally evenly, but sometimes show vacuole-like areas. The filaments do not

stain as deeply as the spores and may show in their interior more deeply staining granules.

Cultures.—The fungus grows on all of the usual culture-media, best in those containing sugar. It is aërobic, and thrives as well at room temperature as in the incubator. The colonies on solid media are at first pale gray or nearly white, and have a delicate fringe of radiating filaments at their margins. Later, they spread widely over the surface of the media and form a layer or a membrane 1 or more mm. thick with wrinkled surface,
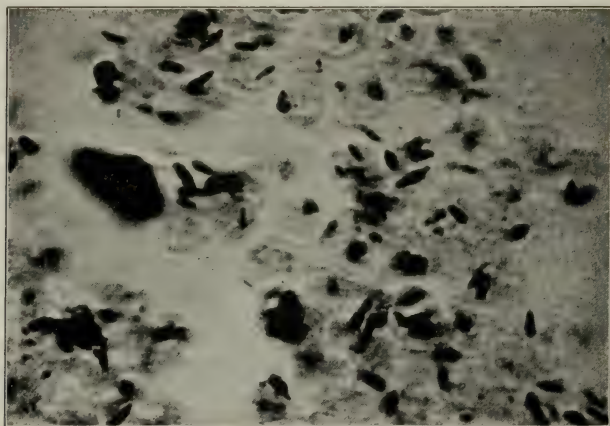
Fig. 112.—Sporotrichum Schenckii. Section of nodule in abdominal wall of white rat. Gram's stain. Cells and spores, the latter oblong and deeply colored; × about 1000 (L. Hektoen and C. F. Perkins).

which may become brown or black in color and of a velvety appearance. Downgrowth may occur into the underlying media. In stab cultures, lateral outgrowths occur along the line of inoculation. Gelatine is slowly liquefied by the micro-organism. In bouillon, growth appears as a downy sediment. The growth in the original cultures from the lesions may be slow, the colonies appearing after a week or more. In succeeding generations the growth is more rapid.

Pathogenesis.—The micro-organism is pathogenic for experimental animals especially for white rats. In the latter animals, septicemia, and disseminated suppurative or granulomatous lesions may be produced by inoculation with cultures. The

testicles are very frequently involved after intraperitoneal inoculation. In the experimental lesions the organism does
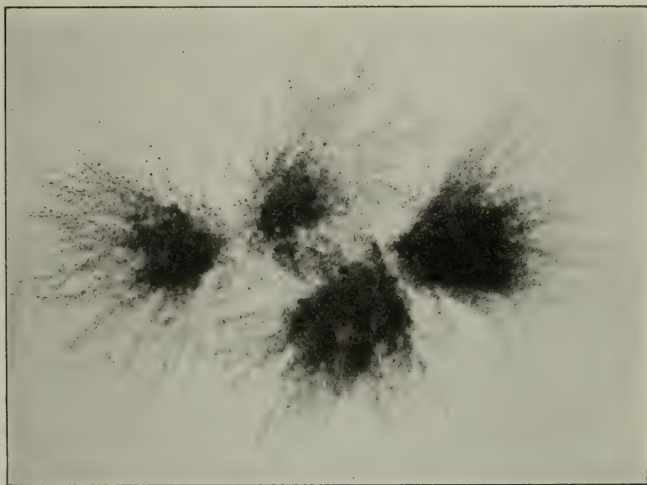
Fig. 113.—Sporotrichum Schenckii. Colonies on glycerin-agar plate. Low power (L. Hektoen and C. F. Perkins).
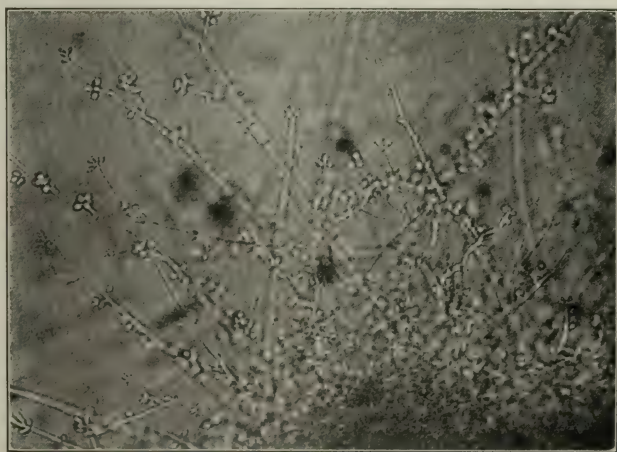
Fig. 114.—Sporotrichum Schenckii. Margin of hanging-drop culture; × about 150 (L. Hektoen and C. F. Perkins).

not appear in the filamentous form, but only in modified forms, more or less resembling the spores and as oblong Gram-staining

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bodies, 1 to 3 microns wide and 10 to 12 microns long. Sometimes small bud-like processes project from these forms.

Occurrence.—The fungus is thought to have a natural habitat in the outer world and to be widely distributed. It is claimed that spontaneous infection with it has been observed in the dog and rat, and that some of the cases of epizoötic or mycotic lymphangitis in horses are due to infection with it.

Diagnosis.—The only practical way by which Sporotrichum Schenckii may be recognized in suspected sporotrichosis is by

Fig. 115.—Sporotrichum Schenckii. Margin of hanging-drop culture; × about 1000 (L. Hektoen and C. F. Perkins).

obtaining it in cultures from unopened gummata or abscesses. As has been pointed out above, it is practically impossible to recognize it in lesions by direct examination.

The Blastomycetes.—Under this heading are included those pathogenic fungi which are regarded as the infectious agents of the granulomatous and suppurative processes, known under the names of blastomycosis, oïdiomycosis, blastomycetic dermatitis, coccidioidal granuloma, and certain others.

Morphology.—In the lesions the micro-organisms appear generally as spherical bodies, each consisting of a protoplasmic mass enclosed in a double-contoured hyaline capsule. The

diameter of the bodies varies up to 30 microns or more. In the protoplasm vacuoles, granules, and various markings may be seen, but no nucleus is apparent. The mode of proliferation in the lesions in the majority of cases is by gemmation or budding. The micro-organisms in some cases have great resemblance to yeast fungi or saccharomyces. In the minority of the known cases proliferation of the micro-organisms in the lesions is not by budding, but by a process which is regarded as one of sporulation, the protoplasm of the larger forms segmenting into
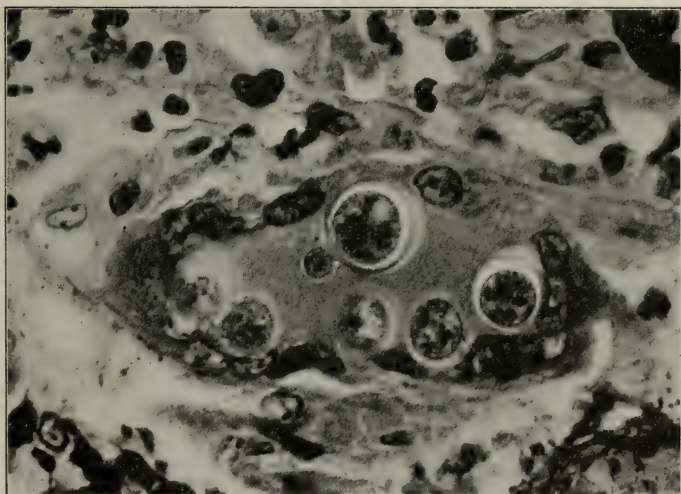
FIG. 116.—Blastomycetes in a giant-cell in the lung. Two of the organisms are budding; × about 1000 (Mallory).

many small spherical bodies. Each of these small spherical bodies acquires a capsule and, being set free by the rupture of the capsule of the mother-cell, develops into an adult parasite.

Cultural Peculiarities.—The micro-organisms grow well at room temperature and in the incubator and upon any of the usual culture-media. According to H. T. Ricketts, the micro-organisms obtained from various cases may be divided into three groups, according to their biological characters, as shown in the cultures as follows:

Group 1.—Those growing chiefly as spherical or oval budding cells and resembling the yeasts, but capable of producing
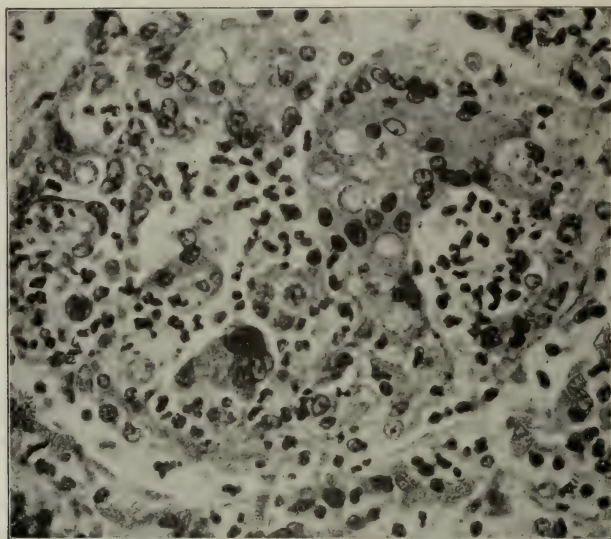
FIG. 117.—Blastomycetes in an alveolus of the lung and the inflammatory reaction caused by them; × about 250 (Mallory).

FIG. 118.—Organism of "Dermatitis Coccidioides." Edge of seventy-two-hour agar colony; × 260 (S. B. Wolbach).

mycelium. The colonies on solid media are elevated, soft, moist, and white, coalescing to form a fleshy growth of paste-

like consistence. Microscopically, the colonies are finely granular. In fluid media, growth appears as a flocculent sediment with a clear supernatant fluid.

Group 2.—Those forming a submerged mycelium which breaks up into chains of spores, while proliferation by budding is not a prominent feature. The colonies at first are granular and slightly elevated. Later they coalesce, become more elevated, and incorporate themselves in the medium.

FIG. 119.—Organism of "Dermatitis Coccidioides." Sprouting sphere at end of twenty-four hours' incubation, showing the development of filaments from it; × 400 (S. B. Wolbach).

Eventually they form a surface like that of a piece of crumpled cloth. Microscopically, the colonies appear as masses of radiating, segmented, and branching hyphæ. In fluid media growth appears as a membrane at the surface with coherent masses or tufts at bottom and sides of the tube.

Group 3.—Those producing mycelia with fruit-bearing aërial hyphæ, and also capable of multiplying by gemmation or budding. The colonies are dry, white, and feathery in appearance, develop hyphæ, which eventually cover the inner surface of the tube. There is growth into the media. Microscopically, the growth is made up of branching, closely segmented hyphæ with many lateral off-shoots. In fluid media the growth

appears as a coherent mycelial tuft at the bottom of the tube with no surface growth.

The micro-organisms of the first and second groups are capable of producing fermentation, while those of the third group are not.
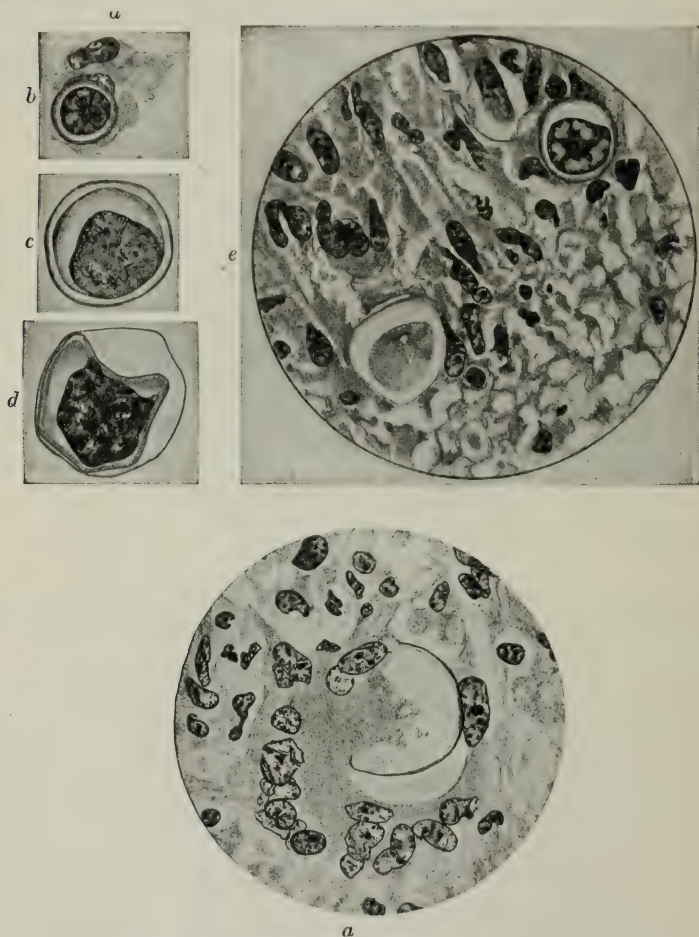
FIG. 120.—Organism of "Dermatitis Coccidioides." Various stages in the development of the organism as seen in tissue (Mallory).

Pathogenesis.—In animals inoculated with cultures of some, but not all, of these micro-organisms, abscesses, granulomatous tumors, and tubercle-like nodules, widely disseminated,

have been produced. In these lesions in the animals the micro-organisms exist in the same form as in the human lesions. Wolbach, working with the micro-organism of the type which proliferates in the tissues by sporulation, and which was not observed to proliferate by budding in cultures, has traced in animals the transformation of the hyphæ of the cultures into the characteristic spherical bodies of the lesions. He found that the spherical bodies arise by the segments of the hyphæ enlarging and assuming spherical shape, the wall of the segment thus becoming the capsule of the spherical body. He also observed pointed and club-shaped hyaline bodies radiately arranged at the periphery and continuous with the capsules of the micro-organisms.

Diagnosis.—The micro-organisms may be easily recognized by mixing a drop of the pus or granulation tissue from a lesion with a little 10 per cent. solution of sodium hydrate and examining the mixture under a cover-glass with the microscope.

ANIMAL PARASITES

Entamœbæ.—Three species of amebæ occur in the intestine. Only one species, *Entamœba histolytica*, is pathogenic and is the cause of amebic dysentery. The others are harmless parasites. Entamœba histolytica is most easily distinguished from the others by its more active movements. In its encysted form it has four nuclei, while one of the non-pathogenic species, E. coli, has eight.

In cases of dysentery suspected of being due to amebæ the stools are best examined as soon as voided, although the amebæ will sometimes remain active in stools even over twenty-four hours old. A warm stage during the examination is an advantage, but not a necessity. A drop of the fluid material, preferably that containing mucus or blood, is placed on a slide and lightly covered with a cover-glass. If the slide is cold and the organisms do not move, warm the slide gently and the movements of the amebæ will often start up. Pus from abscesses due to the amebæ is examined in the same way. The diagnosis is made by finding the characteristic large cell, with single round nucleus, and glassy refringent peripheral cytoplasm which projects itself actively in the form of pseudopods. The cytoplasm often contains vacuoles and red blood corpuscles.

Material obtained with a swab from the margin of ulcers with the aid of the proctoscope is more favorable for the finding of amebæ than material from stools.

Amebæ and their nuclei may be made more clearly visible in wet smear preparations by rubbing up the fecal material or pus with a little solution of iodine, which stains the glycogen present in them, or by mixing with a combination of acetic acid, 10 per cent. formalin and 1 per cent. neutral red solution, each in equal parts.

Permanent stained smear preparations may be made as follows:

1. Make a thin smear and fix, while moist, for fifteen minutes in a mixture of ethyl alcohol 1 part and staturated aqueous solution of corrosive sublimate 2 parts.
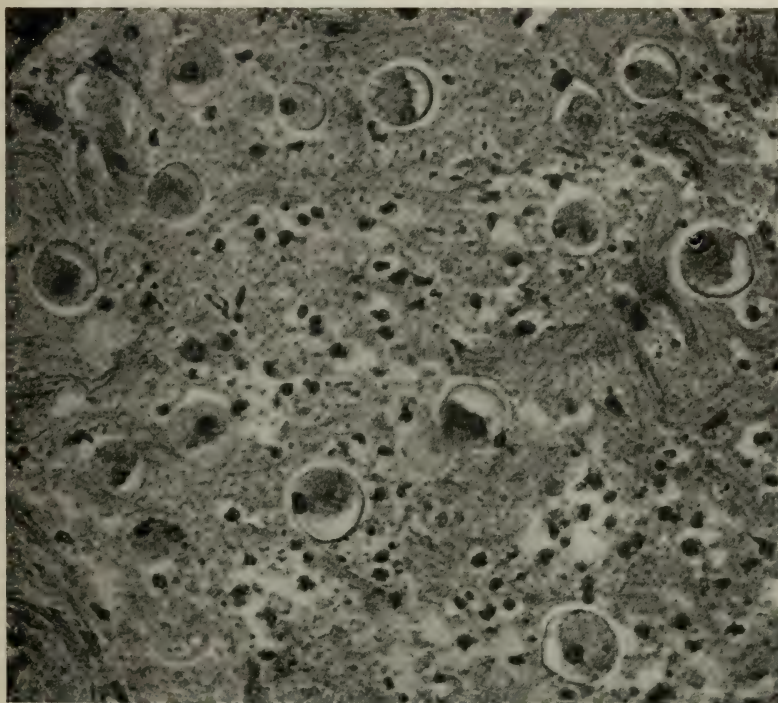
FIG. 121.—Entamœba histolytica in section of ulcer of intestine; × 500 (photo. by F. B. Mallory).

2. Wash in water and cover with 1 per cent. alcoholic solution of iodine for three minutes.

3. Wash with 95 per cent. alcohol until the iodine stain disappears.

4. Wash in water and stain with iron hematoxylin, or phosphotungstic acid hematoxylin, as a section attached to the slide.

5. Dehydrate, clear and mount.

In sections of fixed tissues the nuclei of the amebæ do not stain particularly well with the ordinary nuclear stains, such as alum-hematoxylin and methylene-blue, although phosphotungstic acid hematoxylin brings them out with great sharpness. The following method of staining them has been found to give very satisfactory results and to render the recognition of the organisms easy:

Differential Stain for the Entamebæ (Mallory).—1. Harden in alcohol.

2. Stain sections in a saturated aqueous solution of thionin three to five minutes.

3. Differentiate in a 2 per cent. aqueous solution of oxalic acid for one-half to one minute.

4. Wash in water.

5. Dehydrate in absolute alcohol.

6. Clear in xylol.

7. Xylol-balsam.

The nuclei of the amebæ and the granules of the mast-cells are stained brownish red; the nuclei of the mast-cells and of all other cells are stained blue.

Excellent results were obtained by this method with bits of the purulent discharge from a so-called amebic abscess of the liver. After hardening in 95 per cent. alcohol, small fragments the size of a pin-head and less were stained as above directed, and teased apart after they were in the balsam. The reddish nuclei stood out so sharply in the bluish background of fragmented nuclei and granular detritus that they were easily picked out with the high dry power.

The results obtained with feces examined in the same way or after embedding in celloidin were much less satisfactory, for the reason that various substances in the feces precipitate the thionin in the form of reddish crystals and give rise to deceptive pictures. A similar differential stain can be obtained by Unna's method for staining the granules of mast-cells (see page 117).

Other protozoa, such as the cercomonas and trichomonas, are best examined in fresh preparations.

Malarial Organisms.[1]—Three varieties of the plasmodium malariæ have been described—namely, the tertian, quartan, and estivo-autumnal parasites. They develop within or upon the red corpuscles and cause the destruction of the corpuscles affected. The earliest forms of the parasite appear in the blood during the latter part of the malarial paroxysm or shortly after it. At this time they appear as small, colorless, disc-shaped hyaline bodies which occupy but a small portion of the blood-corpuscles. They possess a varying degree of ameboid movement, the amount depending upon the type of the organism. These ameboid movements are best observed on the warm stage. During the process of development the parasites increase in size and more or less completely fill the red corpuscles containing them. Small particles of reddish-brown pigment are produced, during their growth, from the hemoglobin of the corpuscles in which the organisms are developing. These granules show varying degrees of motion, probably imparted to them by the movements of the parasites. At first the pigment appears to be scattered about in the corpuscle, but it is in reality in the extremities of the pseudopodia. Later it appears more evenly spread about in the periphery. Toward the end of the cycle of development the pigment collects in the center of the parasite; at this time the ameboid movements have ceased, indications of segmentation occur, and the parasite nearly or completely fills the corpuscle. Oftentimes at this stage only a small portion of the corpuscle is visible at some point on the edge of the parasite.

The beginning of segmentation is indicated by a number of radial lines extending from the periphery of the parasite toward the central clump of pigment. Segmentation takes place, and the pigment is surrounded by a number of distinct segments which vary with the type of the organism. Each of these segments shows a central refractive spot which probably is the

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[1] For some important details, here omitted, concerning the morphology and biology of the malarial parasites the reader is referred to the authoritative papers by Mary Rowley-Lawson, *Jour. Exp. Med.*, xiii, p. 263, and by Charles F. Craig, Osler's *Modern Medicine*, i, p. 392.

nucleus.   At this time one notices small hyaline bodies, like those of the early stage in the development of the parasite, in some of the red blood-corpuscles.   Oftentimes such a regular process of segmentation is not observed, but enough has been said to indicate the manner in which reproduction occurs.   Segmentation is the indication of an approach of a paroxysm.   Extra-cellular forms of the parasites are not infrequently seen.   They may be fully grown organisms which have destroyed the corpuscles that contained them, or they may be partly grown organisms which have left the corpuscles.   These free parasites are indistinct in outline and contain pigment.   They possess ameboid movements, and may be considerably larger than a red blood-corpuscle.   Various changes are observed in them:

1. They may increase in size until they become nearly as large as polymorphonuclear leucocytes.   With the increase in size there is a gradual cessation in the movement of the pigment-granules, until finally the organisms present the appearance of misshapen masses of protoplasm containing motionless pigment-granules.

2. They may undergo fragmentation and give off several small circular pigmented bodies.

3. Vacuolization may occur.

4. Flagellate forms may develop.   One or more thread-like processes are thrust out from the organisms.   These flagella may contain pigment, and may break away from the organism and move about among the corpuscles, looking not unlike the spirochetes of relapsing fever.

The three varieties of parasites differ from one another in a number of ways.   The chief differences are the length of the cycle of development; the size of the full-grown organisms; the difference in the refractibility of the organisms; the quantity, size, and color of the pigment-granules; the degree of ameboid movement; and the number and shape of the segments into which the full-grown organisms divide.   In the earliest stage the varieties cannot be distinguished from each other.

The tertian parasite completes its cycle of development in about forty-eight hours. When it has attained its fullest growth it almost fills the corpuscle, which has become larger than normal. This organism is less refractive than either of the other two. The pigment-granules are more numerous, finer, and more reddish-brown in color; the ameboid movements
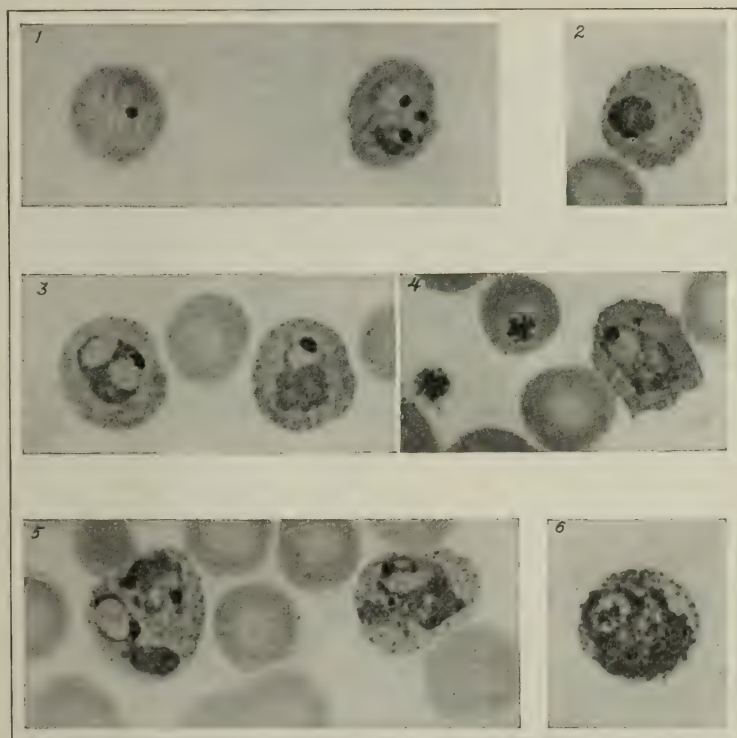
FIG. 122.—Tertian malarial parasites in red blood-corpuscles (Wright's stain): 1, Young parasites (in the corpuscle on the right two or three parasites); 2, young parasites; 3, half-grown parasites; 4, half-grown parasite (on the left, a blood-plate and near the center another blood-plate lying on a red corpuscle); 5, half-grown parasites (in the corpuscle on the left two parasites); 6, full-grown parasite (the nucleus lies in a clear space). All the infected blood-corpuscles in the foregoing figures contain minute granules that stain red (granular degeneration) (photos. by L. S. Brown).

are much more active; the segments are more irregular in shape and more numerous than those of the quartan parasite, varying from twelve to twenty in number.

The quartan appears to complete its cycle of development in from sixty-four to seventy-two hours. The full-grown organism does not fill completely the corpuscle, and the latter is not increased in size. The organism is more refractive than the tertian parasite. The pigment-granules are fewer in number, coarser, and have a darker-red color. The ameboid movements are slower; the segments are pear-shaped, more symmetrical, and less numerous than those of the tertian parasite, varying from six to twelve in number. Segmentary organisms are more numerous in the peripheral circulation than in the case of the tertian parasite.
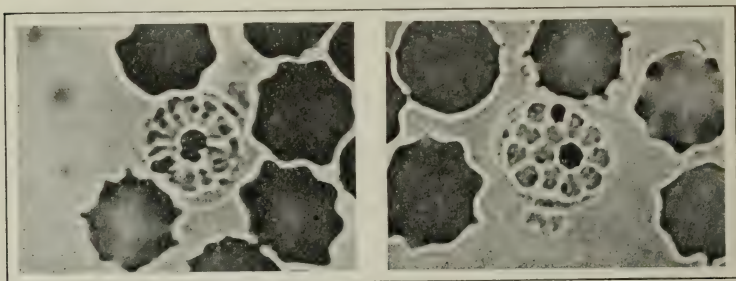
Fig. 122a.—Two stages in the process of segmentation of a tertian malarial parasite in a red blood-corpuscle. Preparation of fresh blood, not stained (Wright and Brown).

The estivo-autumnal parasite cannot be studied so thoroughly in the peripheral circulation, because the latter's development and segmentation take place in the internal organs. The length of time required to complete its cycle of development is not so definitely settled. It appears to require from twelve to twenty-four hours, more or less. The full-grown organism is smaller than the tertian parasite, and the corpuscle which contains it is often smaller than normal and more or less distorted. The parasite is quite refractive. The pigment-granules are few in number and coarse. The ameboid movements are slow. After the duration of fever for from five days to a week or more, elongated, ovoid, or crescent-shaped bodies make their appearance. They are sometimes as large or larger than a red corpuscle. These bodies are
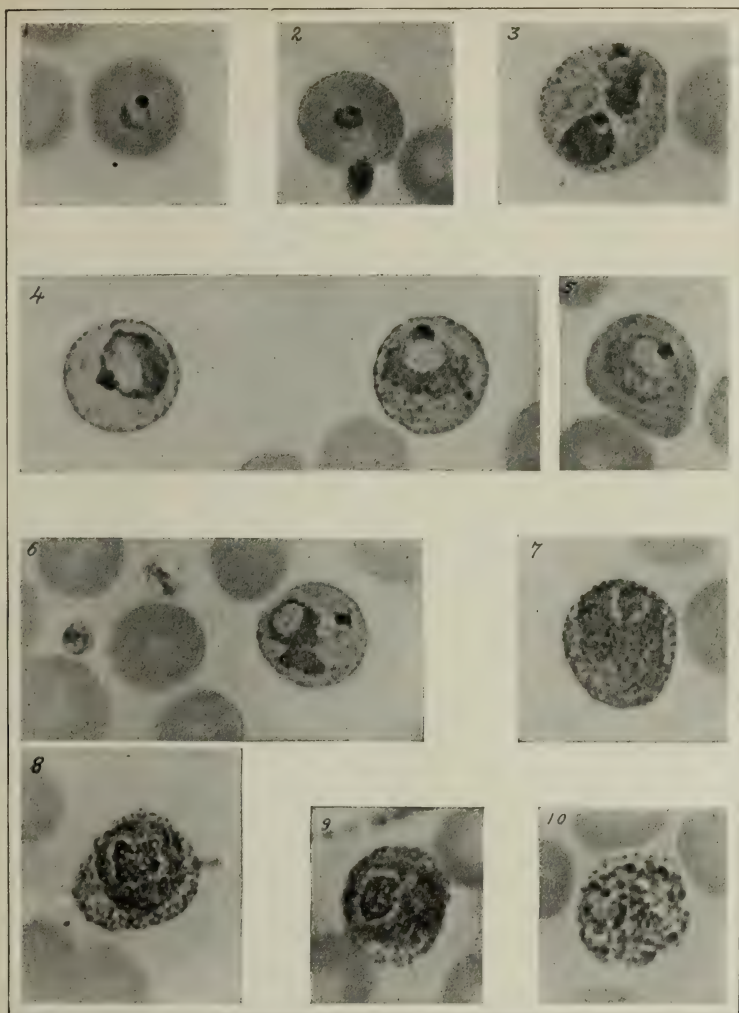
FIG. 122b.—Tertian malarial parasites in red blood corpuscles (Wright's stain): 1, Young parasite; 2, young parasite with blood plate at the margin of the corpuscle; 3, two young parasites in one corpuscle; 4 and 5, immature parasites; 6, immature parasite (on the left two blood plates); 7, adult parasite (chromatin of nucleus in clear space); 8 and 9, adult parasites (the chromatin is the reticular mass near the center of the parasites); 10, segmenting parasite (the chromatin has divided into a number of separate dark colored masses. In the center some pigment). In all of the foregoing figures, except Figure 1, the granular degeneration of the infected red corpuscles is shown (photos. by L. S. Brown).

not a result of segmentation, but appear to be a further develop-
ment of the round hyaline bodies. They are highly refractive
and contain granules of coarse pigment in the center. They
lie usually at one side of the red corpuscles, the latter more or
less completely filling the concavity between the two horns of
the crescent. They may lie in the center of the corpuscles.
Some of the apparently free ovoid bodies are turned in such a
way as to present a convex surface toward the observer.

Double infections occur quite frequently in both tertian
and quartan fever, and in the latter not infrequently triple
infections occur. In the double infections two groups of para-
sites reach maturity on successive days and cause daily
febrile paroxysms. In the triple infection of quartan fever
three groups of organisms mature on successive days and cause
corresponding paroxysms.

Methods of Examining the Blood for Malarial Organisms.—
The organisms of malaria can be detected in fresh specimens
of blood or in specimens of blood which have been fixed and
stained.

In doubtful cases the parasites are more surely and easily
found in cover-glass preparations of the blood fixed and stained
by special methods.

The method employed in making cover-glass preparations
of the blood has been thoroughly described (see preparation
of cover-glass specimens in the Examination of the Blood,
page 470).

Wright's stain for malarial parasites is identical with his
blood-stain and is applied in the same way (see page 471).
It gives the so-called Romanowsky stain to the parasites.

With the stain the body of a malarial parasite stains blue,
while the color of the chromatin varies from a lilac color through
varying shades of red to almost black. In the young forms of
the tertian and estivo-autumnal parasites the chromatin appears
as a spherical, dark red body, while in the older forms of the
tertian parasite it has a more lilac or purplish-red color, and

NOTE.—The description of the development of the parasites is abstracted
from Thayer and Hewetson's *The Malarial Fevers of Baltimore.*

may appear in the form of a reticulum. In the intermediate forms the color of the chromatin may present variations between these extremes (see Fig. 122c).

Blood plates apparently situated within red blood-corpuscles may be mistaken by the inexperienced for young malarial parasites. This ought never to occur if one bears in mind the
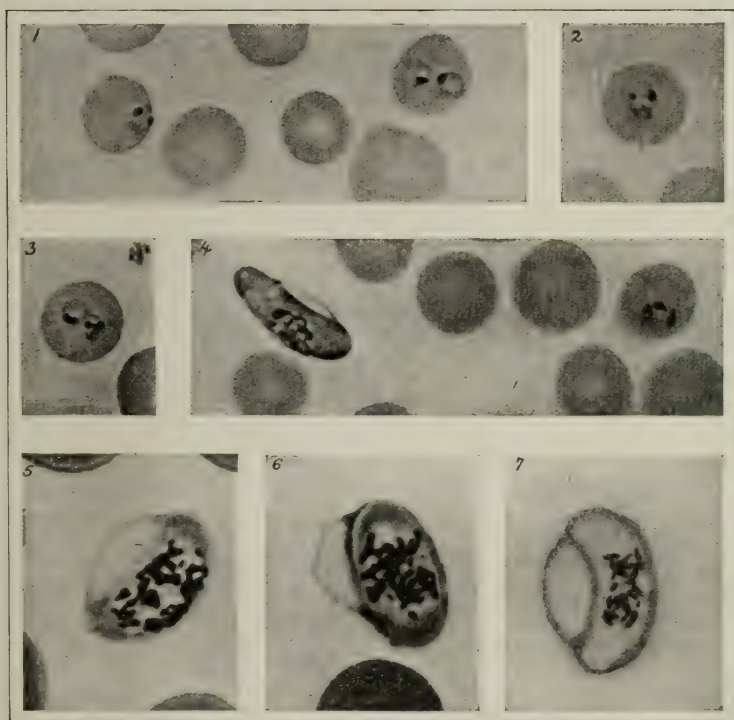
FIG. 122c.—Estivo-autumnal malarial parasites in red blood-corpuscles (1, 2, 3, and 4, Wright's stain): 1, 2, and 4, young parasites; 3, on the left, a "crescent," on the concave side of which is shown a portion of the periphery of the red corpuscle, which it distends; on the right, a young parasite; 5 and 7, "crescents" in red blood-corpuscles; 6, ovoid form of parasite in a red blood-corpuscle (photos. by L. S. Brown).

fact that the young parasite of all the three kinds should present by this method a dark red, spherical nucleus and a homogeneous blue cytoplasm which is usually in the form of a definite ring (see Fig. 122c).

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Various workers have shown by their modifications of the Romanowsky method that red blood-corpuscles harboring malarial parasites have dark red staining granules. These granules may be brought out by the present method, but in order to bring them out, it may be necessary to allow the staining fluid, after the addition of the water to it, to remain on the preparation for at least five minutes, and then not to decolorize or differentiate with water for as long a time or to such an extent as for ordinary blood preparations.

A method described by Ross permits a relatively large amount of blood to be examined and is as follows: A large drop of blood is spread on a slide over an area of that of a cover-glass, allowed to dry and warmed over a flame to fix it. It is then covered with 10 per cent. aqueous solution of eosin for fifteen minutes, after which it is washed in water and covered with a dilute solution of alkaline methylene blue for some seconds, when it is again washed in water, dried and mounted. The red blood corpuscles are eliminated by this procedure and thus do not obscure the blue stained parasites.

In examining a fresh specimen of the blood for the malarial organisms a glass slide is substituted for one of the cover-glasses, and the cover-glass which has the drop of blood on its surface is dropped lightly upon the glass slide and allowed to remain there. The first four or five drops of blood should be quickly wiped away from the ear until a very small drop is obtained. Great care must be exercised to touch only the tip of the drop with the cover-glass, so as to avoid smearing the blood. If the blood is smeared on the cover-glass, the edges of the blood-drop will dry before the cover-glass can be transferred to the slide, and the blood will not spread. It is necessary that the blood should spread in a thin layer in order to study satisfactorily the individual corpuscles. If one desires to study the preparation for several hours, the edges of the cover-glass can be surrounded by melted paraffin or vaselin to exclude the air. The examination should be made with an oil-immersion lens. It should be remembered that the action of cold inhibits the ameboid movements of the parasites; it may be necessary,

therefore, at times to warm the slide before examining the specimen. Evaporation not infrequently occurs, caused by the air penetrating beneath the cover-glass. This produces changes in many of the corpuscles which may be mistaken for hyaline bodies: the central depression becomes paler and less refractive than the periphery of the corpuscles; later a number of corpuscles contain small glistening points, and still later the corpuscles become crenated.

Giemsa's Stain.—This also gives the Romanowsky staining. The formula is as follows:

Azur II—eosin,	3	gm.;
Azur II,	0.8	"
Glycerin (Merck, chemically pure),	250	"
Methyl-alcohol (Kahlbaum I.),	250	"

The staining fluid is manufactured by Grübler, and it is best to obtain it already prepared.

1. The preparation is dried in the air and fixed in absolute alcohol fifteen minutes, or in methyl-alcohol two or three minutes, after which the alcohol is removed with filter-paper.

2. To 1 c.c. of distilled water in a small graduate add 1 drop of the staining fluid and skake gently. This dilution is to be made immediately before proceeding to the next step.

3. Cover the preparation with freshly diluted staining fluid for ten to fifteen minutes.

4. Wash in a stream of water.

5. Remove excess of water with filter-paper, dry in the air, and mount in balsam.

If specially intense staining is desired, add to the distilled water before mixing it with the stain a little potassium carbonate solution in the proportion of 1 or 2 drops of a 1 per cent. solution to 10 c.c. of water.

Giemsa's Method for Staining Protozoa and Bac= teria in Sections.—1. Fix pieces of tissue not more than 2 mm. thick in sublimate alcohol, consisting of 2 parts of a concentrated aqueous solution of corrosive sublimate and 1 part of absolute alcohol. The fixation requires at least forty-

eight hours. The fixing fluid is to be renewed after twenty-four hours.

The tissue may remain for as long as three months in the fixing fluid without disadvantage if evaporation is prevented.

2. Dehydrate in graded alcohols and xylol. Embed in paraffin. The sections should not be over 4 microns thick—two microns are better. The tissues must not be handled with metal instruments until after they have been cleared in oil of cedar wood.

3. Treat sections with xylol, followed by graded alcohols and water.

4. Ten minutes in a solution consisting of KI, 2 gms.; distilled water, 100 c.c.; Lugol's solution, 3 c.c.

Instead of this mixture, it is possible to use Lugol's solution only (1 to 3 c.c. of it mixed with 100 c.c. of water or 70 per cent. alcohol), or tincture of iodin diluted with alcohol. The use of the weak alcoholic iodin solution is indicated when a more intense blue staining of the cytoplasm is desired. Treatment with the weaker iodin solutions demands naturally a longer time—twenty to thirty minutes.

5. Treat with 95 per cent. alcohol until the yellow color is removed. After a quick wash with distilled water place sections for ten minutes in a 0.5 per cent. aqueous solution of sodium hyposulphite, then five minutes in tap-water, and for a short time in distilled water.

6. Stain with freshly diluted Giemsa solution two to twelve hours or longer. The solution recommended for this purpose should be made up according to the following modified formula:

Azur II—eosin,	3 gm.;
Azur II,	0.8 "
Glycerin,	125 c.c.;
Methyl-alcohol,	375 "

The dilution should be 1 drop to 1 c.c. of water; or for a longer period of staining, 1 drop to 2 c.c. of water. After the first half hour the staining mixture is to be poured off and replaced by fresh.

7. Wash in distilled water and dehydrate as follows:

(*a*) Acetone 95 c.c. plus xylol 5 c.c.
(*b*) Acetone 70 c.c. plus xylol 30 c.c.
(*c*) Acetone 70 c.c. plus xylol 30 c.c.
(*d*) Xylol pure.
(*e*) Cedar oil.

8. Mount in cedar oil.

The duration of the treatment with *a*, *b*, and *c* depends upon the degree of differentiation required.

The distilled water used for diluting the staining fluid must be absolutely free from acid. The slightest trace of organic or mineral acids, or even the presence of a considerable amount of carbonic acid, spoils the staining. The distilled water may be tested and corrected for use as follows:

Place 300 c.c. of it in each of 4 flasks. Add 1 per cent. solution of carbonate of sodium (Na_2CO_3), 1 drop to the first flask, 2 drops to the second flask, and so on. Then take 10 c.c. from each flask in a clean test-tube and add 2 or 3 drops of a fresh solution of hematoxylin in absolute alcohol, which should be pale yellow to nearly colorless. Stand against a white background, and that flask with the right reaction should take on a violet tinge after one to five minutes.

For bringing out certain granules, etc., in special objects a larger amount of alkali in the water is necessary. In this case add to 20 c.c. of the water, shortly before mixing with the staining fluid, an additional drop of alkali solution.

S. B. Wolbach suggests, after considerable experience with the method, the following modifications:

2. Clear in cedar oil instead of xylol.

4. Use the weak solution of iodin in 70 per cent. alcohol.

6. The dilution should be 60 drops of the stain to 100 c.c. of distilled water to which has been added 10 c.c. of methyl alcohol and 2 drops of a 0.5 per cent. solution of sodium carbonate. The stain is replaced twice by fresh mixtures during the first hour.

7. Transfer the sections for differentiation directly from the staining mixture or, after a rapid passage through distilled water, into two changes of the following solution:

Colophonium,	15 grams;
Acetone,	100 c.c.

The slides should be treated individually, and the differentiating fluid should be renewed as soon as the colophonium precipitated by the water fails to dissolve quickly. Differentiation takes place rapidly, fifteen to thirty seconds are usually sufficient. Should a deeper staining with blue be desired the amount of colophonium should be reduced.

8. Pass the sections rapidly through—

Acetone,	70 c.c.;
Xylol,	30 "

followed by pure xylol and then by oil of cedar wood. Mount in oil of cedar wood.

Rabies (Hydrophobia).—The diagnosis of this disease from a pathological standpoint may be made by the production of experimental rabies in a rabbit by intradural inoculation with material from the nervous system of the animal suspected to have died of it. The etiological agent of the disease occurs in the brain, spinal cord, salivary glands, and pancreas. For purposes of inoculation a piece (1 or 2 c.c.) of the medulla or brain, preferably the former, is rubbed up in a sterilized mortar with about 10 c.c. of sterilized distilled water. The resulting fluid is filtered through absorbent cotton, and then through filter-paper, to remove tissue-shreds. Of the clear fluid thus obtained 4 or 5 drops are injected beneath the dura of a rabbit by means of a hypodermic syringe, the skull being trephined with a small trephine about 4 mm. in diameter. The most favorable place for opening the skull is at a point in the median line just posterior to a line drawn through the middle of each eye.

The symptoms of experimental rabies in the rabbit first manifest themselves after two weeks, never earlier, but they may not appear until later, not even until two months have passed. The first symptom is a weakness of the hind legs, followed by paralysis. The paretic condition soon extends to the fore legs, dyspnea appears, and death usually occurs in about three days after the onset of the symptoms. Paralytic symptoms developing before two weeks are not due to infection with rabies, but to some other cause; for instance, infection with the pneumococcus or other bacteria which may be present in the material inoculated.

During the course of the disease the animal never appears stupid, with dull eyes, as in other infections, but remains "conscious," so to speak, until the last.

The diagnosis of rabies is made by finding in the nerve-cells of the central nervous system peculiar bodies which are regarded as protozoa, and which are known as "Negri bodies," from the name of their discoverer. These bodies are generally round or oval, but may be irregular, pear-shaped, or triangular in form. They vary in diameter up to 23 u. They contain small vacuoles, in some of which are granules of varying size and number; generally there is a central larger structure surrounded by smaller ones. In preparations stained by the eosin-methylene-blue method the bodies generally stain deeply with eosin, with the exception of the granules, some of which stain with the methylene-blue.

Method of Demonstrating Negri Bodies.—The bodies may be sought for in smear preparations or in sections. Pieces of gray brain-substance should be taken for examination from the cortex in the region of the fissure of Rolando (in the dog from around the crucial sulcus), from the hippocampus, and from the vermes of the cerebellum.

For demonstrating the bodies in sections the tissue should be fixed in Zenker's fluid, embedded in paraffin, and may be stained by the eosin-methylene-blue method. A. E. Steele obtained good results by staining the sections with carbol-fuchsin solution, mordanting and decolorizing with undiluted

formalin, and then after washing in water, counterstaining with methylene blue. The Negri bodies are stained purple red and the cell nuclei blue.

For demonstrating the bodies in smear preparations the following procedure is said to give the best results: A small bit of the gray substance of brain chosen for examination is cut out with a small, sharp pair of scissors and is placed about 1
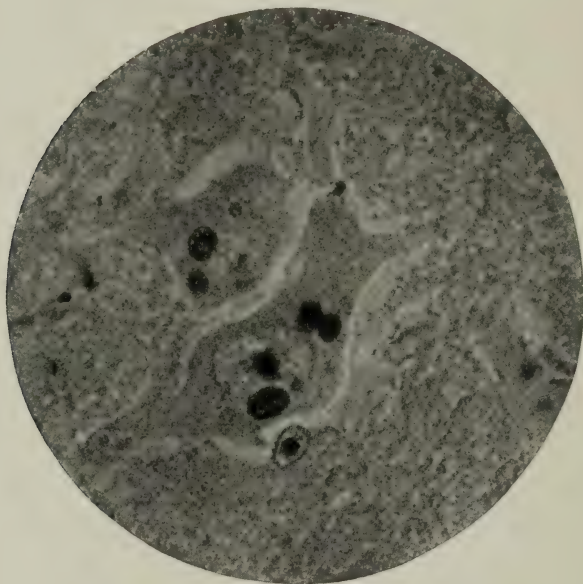
Fig. 123.—Ganglion-cells containing Negri bodies.

inch from one end of a slide. The cut in the brain should be made at right angles to the surface and a thin slice taken, avoiding the white matter as much as possible. A cover-slip is now pressed down upon the piece of tissue until it is spread out in a moderately thin layer, then the cover-slip is moved slowly and evenly over the slide to its other end. The preparation is then partially dried in the air and fixed in methyl-alcohol for about five minutes. It may be stained by Frothingham's mixture, which is:

Saturated aqueous solution of methylene blue, 4 drops;
Saturated alcoholic solution of basic fuchsin, 6 "
Water, 30 c.c.

or by Mann's mixture, which is:

1 per cent. aqueous solution of methylene blue, .4 c.c.;
1 per cent. aqueous solution of eosin, .2 "
Distilled water, 3.0 "

These mixtures are to be freshly made. To obtain satisfactory results with them it may be necessary to vary the proportions of the ingredients and the time of staining.

Spirochetes of Relapsing Fever.—They are present in varying numbers in the circulating blood before and during the
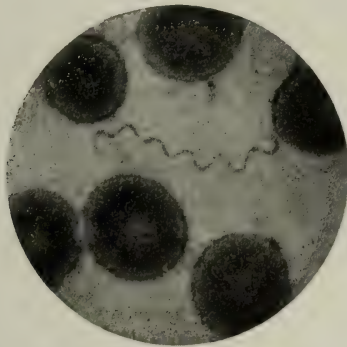
Fig. 124.—Spirochetes of relapsing fever. Smear preparation from blood; stained by Wright's blood-stain. × 1500 (photo. by L. S. Brown).

febrile paroxysms and may be demonstrated in cover-glass preparations by the methods described for staining malarial parasites.

Syphilis.—**Methods of Demonstrating Treponema Pallidum (Spirochæta Pallida) in Smear Preparations.**—The lesions are to be cleansed from any adherent exudate. The smear preparations are to be made from the juice of the tissue obtained by pressure and scraping. An excess of blood should be avoided. The preparations are then dried in the air and may be stained by the following methods:

1. *Method with Wright's Blood Stain.*—Place in a test-tube 10 c.c. of distilled water, 1 c.c. of the blood-staining fluid, and 1 c.c. of a 0.1 per cent. solution of potassium carbonate. Heat to boiling and cover the preparation with the hot mixture. After three or four minutes, when the fluid on the preparation has become of a violet color and a thin yellow metallic scum has formed on the surface, pour off and again cover the preparation with the hot mixture after again heating in the tube. Repeat this once more. Dry and mount.
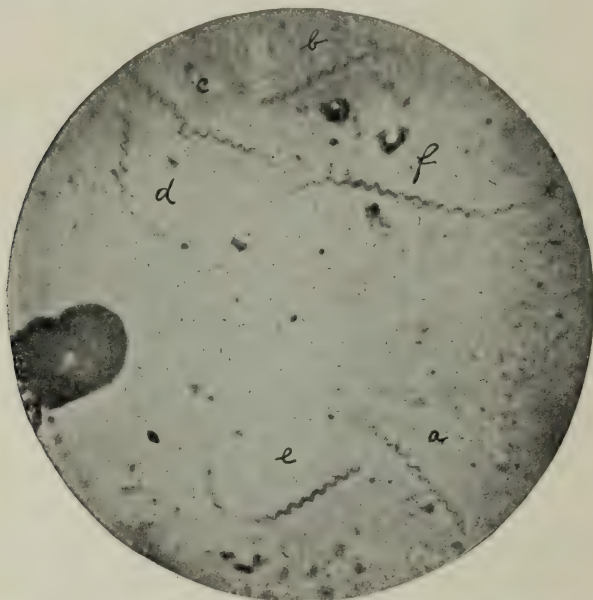
FIG. 125.—Treponema pallidum in smear preparation (Goldhorn).

The material should be thinly spread on a cover-glass, not on a slide, the cover-glass to be held level with forceps during the staining.

The treponemata should stain intensely violet.

2. *Giemsa's Method.*—This is the same as his method for staining malarial parasites (see page 435), except that the preparation is fixed in absolute alcohol for fifteen to twenty minutes, and that to the water used for diluting the staining fluid 1 to 10 drops of a 0.1 per cent. solution of potassium

carbonate is added.   Preparations which are overstained may
be differentiated by washing in distilled water for one to fifteen
minutes.

Recently it has been shown that heating the diluted stain-
ing fluid on the preparation stains the treponemata much
more intensely.   This modified method is as follows:

Ten drops of Giemsa's staining fluid are mixed by gently
shaking with 10 c.c. of distilled water immediately before
proceeding to the staining.   The preparation is fixed in abso-
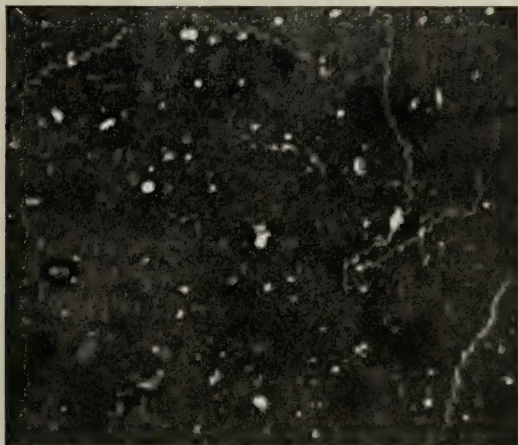
Fig. 126.—Treponema pallidum in a smear preparation stained by Benian's
method; × 1500 (Wright and Brown).

lute alcohol fifteen minutes, or by drawing three times through
the flame.   It is then covered with the diluted staining fluid
and warmed until a slight steam arises over the flame, and
allowed to cool about fifteen seconds, when the diluted staining
fluid is poured off and replaced by fresh fluid, and this again
warmed to steaming and allowed to cool for about fifteen
seconds.   This process is repeated four or five times, after
which the preparation is washed, dried, and mounted in balsam.
In this modified method the staining of the parasites is intensely
dark red.   It is important that the slide or cover-glass be free
from grease, and that the test-tube and the cover-glass or slide
forceps be clean, free from acid, and from any precipitated stain.

3. *Benian's Method.*—Thoroughly mix on a cover-glass one or two loopfuls of 2 per cent. aqueous solution of Congo red with a small amount of serum or exudate from the lesion. Spread the viscid mixture evenly and rather thinly and dry in the air; then wash with 1 per cent. aqueous hydrochloric acid, drain off at once, and again dry in the air. Do not wash in water or blot.

The spirochetes appear white and unstained in a blue ground, which should be homogeneous. Too large a proportion of exudate results in an unsuitable granular ground.

Serum or exudate which has been preserved by drying on glass is available for use by this method.

4. *Fontana's Method.*—1. Dry the smear preparation in the air without heating.

2. Wash several times with a mixture consisting of 1 c.c. of glacial acetic acid, 20 c.c. of formalin, and 100 c.c. of distilled water.

3. Wash in water and cover with a 1 per cent. aqueous carbolic acid solution in which 5 per cent. tannic acid has been dissolved. Heat until steam arises and allow to cool for thirty seconds.

4. Wash in water, cover with the silver solution described below, heat until steam arises, and allow to cool for thirty seconds.

5. Wash, dry, and mount.

The spirochetes appear brown to black.

The silver solution must be freshly prepared and is made by adding dilute ammonium hydrate solution drop by drop to a $\frac{1}{4}$ per cent. solution of silver nitrate in distilled water until a faint turbidity appears. Excess of ammonium hydrate must be avoided, for this clears up the turbidity, and such a solution is not suitable for use.

5. *Ghoreyeb's Method.*—In this method the following solutions are used:

1. One per cent. aqueous solution of osmic acid.

2. Liquor plumbi subacetatis, diluted one hundred times with distilled water. This diluted solution should be freshly prepared.

3. Ten per cent. aqueous solution of sodium sulphid. A thin smear is preferable. No heat fixation is necessary.

The smear is stained as follows:

1. Cover with osmic acid solution for thirty seconds.
2. Wash in water.
3. Cover with lead subacetate ten seconds.
4. Wash in water.
5. Cover with sodium sulphid solution ten seconds.
6. Wash in water.

This process is gone through with three times. Following this the osmic acid solution is applied for thirty seconds, and
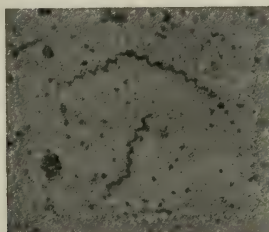
FIG. 127.—Treponema pallidum; smear preparation from a cutaneous papule; stained by Ghoreyeb's method; × 1500 (photo. by L. S. Brown).

the specimen is then washed in water, dried, and mounted in balsam. A thorough washing in running water is essential after the application of each solution to prevent the formation of excessive precipitates.

The osmic acid, the first time applied, acts as a fixative and a mordant. The lead unites with the albumin to form lead albuminate, a compound insoluble in water. The sodium sulphid transforms the lead albuminate into lead sulphid, and causes the preparation to become stained brown. The osmic acid turns the brown color to black. The spirochetes, bacteria, and cellular detritus are stained black.

6. *India Ink Method of Burri.*—Approximately equal parts of the juice from the lesion and of fluid India ink are quickly mixed together on a slide with the aid of a platinum loop, spread thinly, and allowed to dry thereon. When dry, the preparation is ready to be examined directly with the oil-

immersion objective without covering it with balsam and a cover-glass. If the material contains many cellular elements or detritus, it will be necessary to dilute the ink with water. The preparation should have a brown color. The spirochetes and bacteria appear as unstained bodies in a brown to black background.

Some specimens of fluid India ink are said to contain spirochete-like bodies, and therefore the ink used should be known to be free from such.

7. *Levaditi's Method for Staining Treponema Pallidum in Sections.*—1. Pieces of tissue about 1 mm. thick are placed in 10 per cent. formaldehyde for twenty-four hours.

2. Rinse in water and place in 95 per cent. alcohol for twenty-four hours.

3. Place in distilled water until the tissue sinks to the bottom of the container.

4. Place in a 1.5 or 3 per cent. solution of nitrate of silver and keep in the incubator at 38° C. for three to five days. The stronger solution of nitrate of silver is preferable for tissues removed during life.

5. Wash in distilled water and place in the following solution for twenty-four to seventy-two hours at room temperature:

Pyrogallic acid,	2–4 gm.;
Formaldehyde,	5 c.c.;
Distilled water,	100 "

6. Wash in distilled water.

7. Dehydrate in alcohol, clear in chloroform, and embed in paraffin in the usual manner.

The treponemata are stained intensely black by the precipitation of metallic silver upon them and the tissue yellow to brown. The sections may be counterstained with Giemsa's blood stain, but this is of doubtful advantage.

Levaditi and Manouélian's Method.—For demonstrating Treponema pallidum in the meninges and central nervous system, Jahnel has used the following modification of this method:

1. Cut blocks of tissue from old formalin-fixed tissue and place in alcohol for one to three days.

2. Wash in water until the block sinks.

3. Place the block in 1.5 per cent. aqueous solution of silver nitrate in a dark flask at 37° C. for five to eight days.

4. Wash the block in distilled water for two hours.

5. Place the block in Levaditi-Manouélian developer for one day. This developer is made by mixing immediately before use 90 c.c. of a fresh aqueous solution of pyrogallic acid and 10 c.c. of acetone, and, after pouring off 15 c.c. of the mixture, adding or substituting 15 c.c. of pyridin.

6. Embed in paraffin and cut sections.

Wright's Method for Staining Treponema Pallidum in Sections.

1. Fix the tissue with formaldehyde and cut sections by the freezing or the paraffin method. Paraffin sections are to be attached to the slide.

2. Immerse the section in 15 or 20 c.c. of 0.02 per cent. aqueous solution of nitrate of silver, and place in the paraffin oven at 54° C. for thirty minutes.

3. Transfer the section to 15 or 20 c.c. of hot reducing or "developing" solution, consisting of:

Gallic acid,	.5 grams;
Sodium acetate,	5.0 "
Distilled water,	320.0 c.c.

This solution is to be heated to boiling immediately beforehand.

4. Transfer the section to 15 or 20 c.c. of 0.02 per cent. hot aqueous solution of nitrate of silver (which has been heated to boiling immediately beforehand) for thirty seconds.

5. Transfer the section back again to the still hot reducing solution for thirty seconds.

6. Wash the section in distilled water, dehydrate with alcohol, clear with xylol and mount.

The dehydrating, clearing and mounting of frozen sections is facilitated by attaching them to the slide, after staining, by the aniline-clove oil method (see page 54).

In the case of paraffin sections attached to the slide superfluous solutions of nitrate of silver and of "developing" solution should be removed with a towel from the slide and from around the section before transfer from one solution to the other.

It is convenient to boil the solutions in test tubes and to pour them into small liqeueur glasses, in which the sections are treated. All glass ware should be clean.

This method gives essentially the same microscopic pictures as the Levaditi method.

Blocks of tissue which have remained in formaldehyde solution longer than is necessary for proper fixation, undergo a peculiar change, progressing from the surface inward with time whereby the tissue becomes spoiled for staining by this method. This change may be prevented and arrested by transferring the tissue, after fixation, to ethyl alcohol, in which it keeps indefinitely. Before cutting frozen sections of such tissue, the alcohol must be removed by soaking in water.

Infections with Worms.—An important means of diagnosis of worm infection and of the kind of worm concerned is the finding and identification of their ova or larvæ in the feces by microscopical examination. It is generally desirable to examine as large a sample of the feces as is practicable, for the ova and larvæ may be so few as to escape detection in smears made directly from the feces. Several methods are available for doing this.

Barber's Method.—Mix thoroughly a quantity of the feces with equal parts of glycerine and a saturated solution of magnesium sulphate in water. By reason of the high specific gravity of the mixture, the ova and larvæ float and concentrate at the surface, and may be poured off onto slides where they may be sought for with the microscope. In some cases where the eggs are very few, they may be found by centrifugating the upper layers of the mixture and proceeding as before.

Kofoid and Barber's Method.—Mix the feces thoroughly with concentrated urine in a cylindrical container, and push to the bottom the debris, which has risen and is floating at the surface, by means of a straining disk of No. 9 steel wool. The mixture is

then allowed to stand an hour for the ova and larvæ to float to the surface. Then the surface film is removed to slides with wire hooks $\frac{1}{2}$ inch in diameter and examined microscopically.

Tape=worms.—It is not always easy to recognize the kind of tape-worm by a single segment passed with the feces, because
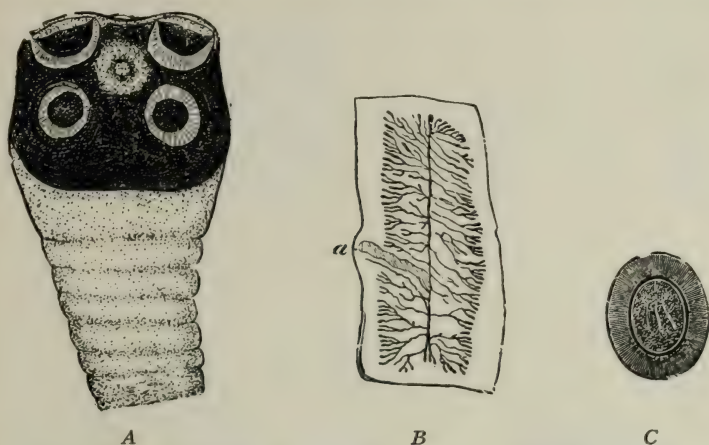
FIG. 128.—Tænia mediocanellata: *A*, head darkly pigmented; *B*, ripe joint, × 6; *C*, egg of tænia mediocanellata.

. the uterus, which furnishes the most characteristic points of difference, is not developed in the young segments and is atrophied in the old ones. When the whole worm is obtained

FIG. 129.—Echinococcus: scolices, hooks (Heller).

the problem is much simpler. The uterus is best made out by squeezing a segment between two slides and holding it up to the light. The heads are examined under the microscope in water, salt solution, or glycerin.

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Tænia Solium (Fig. 142).—Head has four suckers and a circle of hooklets; uterus is noticeably but little branched. The genital tract opens laterally. The eggs develop into the
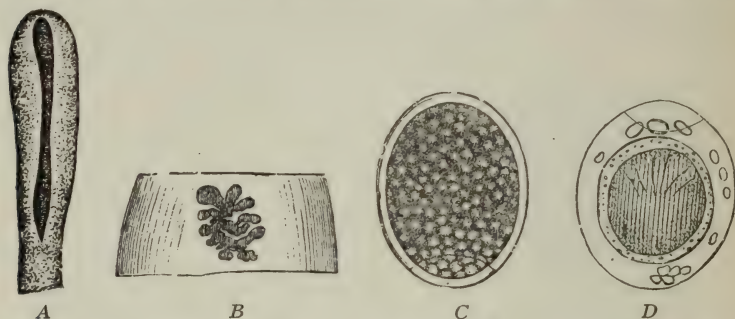
FIG. 130.—Dibothriocephalus latus: *A*, head; *B*, ripe joint, × 6; *C*, egg of dibothriocephalus latus (Heller); *D*, egg with developed embryo (Leuckart).

cysticerci cellulosæ, which are not infrequently found in man. The scolex is obtained for examination by tearing open the

FIG. 131.—Segments of tænia saginata (after Stein).

FIG. 132.—Segments of bothriocephalus latus (after Stein).

FIG. 133. — Segments of tænia solium (after Stein).

cyst and examining the inner wall. The suckers and hooklets are best studied after mounting fresh and pressing under a cover-glass.

Tænia Mediocanellata s. Saginata (Fig. 128).—Head has four strong suckers, but no hooklets; uterus is very much branched, segments show marked muscular development. The genital tract opens laterally. The eggs develop into *cysticerci*, which do not occur in man.

Tænia Echinococcus (Fig. 129) occurs in dogs. The echino-coccus cysts which occur in man are recognized by the very characteristic laminated structure of the cyst-wall. The heads of the scolices have four suckers and a double circle of hooklets.

Dibothriocephalus latus (Fig. 130).—The opening of the genital tract lies in the median line. The head is flattened, and has two small suckers situated at the sides.
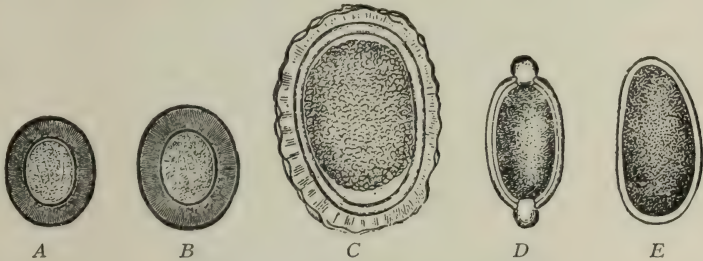
FIG. 134.—Comparative size of eggs of intestinal parasites: *A*, tænia solium; *B*, tænia mediocanellata; *C*, ascaris lumbricoides; *D*, trichocephalus dispar; *E*, oxyuris vermicularis (after Strümpell).

Schistosoma Hæmatobium (Distomum Hæmatobium, Bilharzia).—The male and female parasites occur in the branches of the portal system, especially in the veins of the bladder and rectum, and in the liver. The ova escape from the blood-vessels into the bladder and occasion violent inflammation. The process may extend to the kidneys. The ova also infect the rectum, causing a sort of dysentery, and may involve even the appendix. The ova, with their pointed spines, are characteristic, and may be found by microscopical examination in the urine and feces. The spines are usually situated at one end, but may occur anywhere in the periphery.

Round=worms.—The embryos of the *filaria sanguinis hominis* or *filaria Bancrofti* (Fig. 137) are examined for in

suspected cases by mounting a drop of the fresh blood or of the chylous or bloody urine on the slide and examining

FIG. 135.—Schistosoma hæmatobium. Ovum from fæces showing pointed spine on one side; × 300 (photo. by L. S. Brown).

under low power. They are readily detected when present on account of their very active movements. Six species
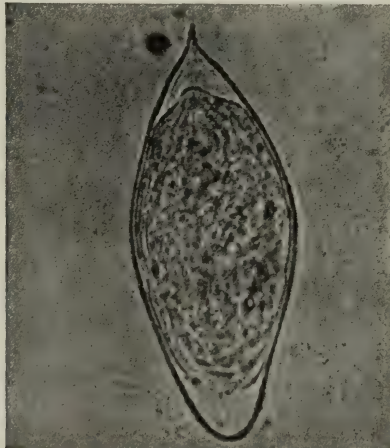
FIG. 136.—Schistosoma hæmatobium. Ovum from urine showing terminal spine; × 375 (photo. by L. S. Brown).

have been described, but the filaria nocturna is the only one that is known to be pathogenic. The blood should be examined

during the resting hours of the patient, as at night for day-workers and during the day for night-workers. Permanent
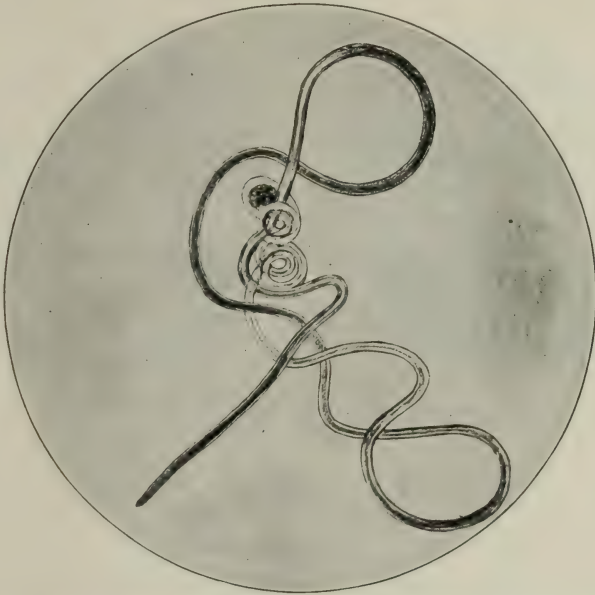
FIG. 137.—Adult male filaria Bancrofti; × 10 (after Lothrop and Pratt).

specimens can be made by fixing ordinary cover-slip preparations of the blood or chylous fluid by heat or by the use of a saturated solution of corrosive sublimate, and staining for a few seconds

FIG. 138.—Photomicrographs of living filariæ sanguinis hominis; × 250. *a*, from hydrocele fluid; *b*, from blood (after Lothrop and Pratt).

with Löffler's methylene-blue or with a 2 per cent. aqueous solution of thionin.

Anchylostoma Duodenale and Necator Americanus ("*Hook Worm*").—The eggs occur in the feces and their recognition may be facilitated by the methods given elsewhere (page 448).
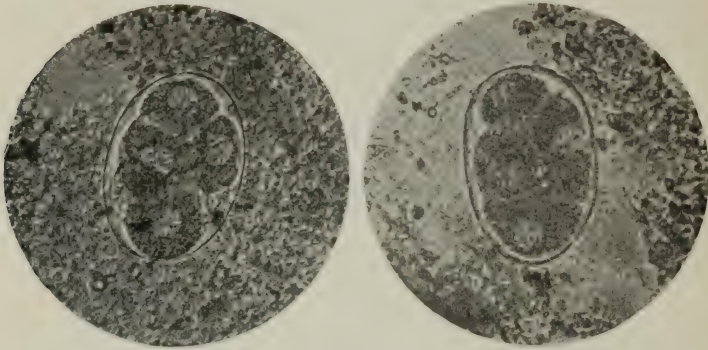
FIG. 139.—Two eggs of the hookworm in feces, each containing several embryonic cells; × 375 (Dr. C. L. Overlander).

The adult worm may be found in the feces after the administration of an anthelmintic.

Trichinellæ (Figs. 140, 141) are obtained from the fresh muscle by means of teasing. A quick method is to squeeze

FIG. 140.—Living embryos (Heller).
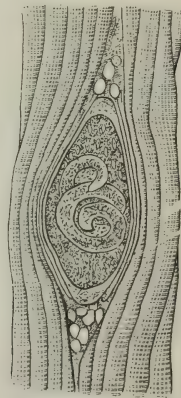
FIG. 141. — Encapsulated trichina (Leuckart).

small bits of tissue between two slides and examine with a low power. Pieces of muscle nearest the insertion of the tendon are chosen from the diaphragm or from the muscles of the jaws.

Encapsulated and calcified trichinellæ are cleared up by means of acids.

In hardened tissues the trichinellæ are best studied in longitudinal sections of the muscle-fibers.
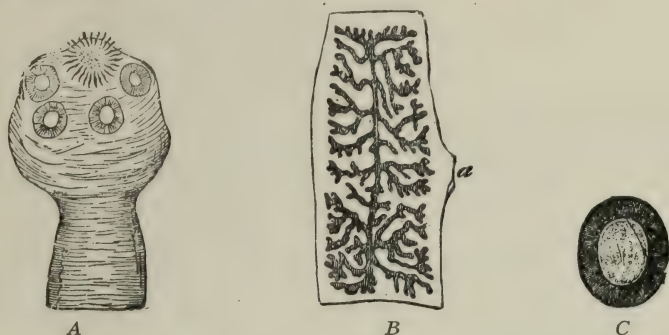
FIG. 142.—Tænia solium: *A*, head enlarged; *B*, ripe joint, × 6; *C*, egg of tænia solium (Heller).

The other round-worms which sometimes occur in the intestinal tract can be recognized with the naked eye. Their eggs must be looked for with the microscope.

The embryo trichinellæ may be demonstrated in the blood by withdrawing some blood with a syringe from a vein in the arm, washing it with 3 per cent. acetic acid, centrifugalizing, and examining the sediment (W. H. Herrick and T. C. Janeway).

THE BLOOD[1]

CONTENTS

[1] This section has been rewritten by Dr. Thomas E. Buckman.

2. Estimation of blood volume.
3. Study of the resistance of the red corpuscles.
    (a) Technique of determining the resistance of the red corpuscles to hypotonic sodium chloride solution.
    (b) Technique of determining the resistance of the red corpuscles to hypotonic blood serum.
    (v) Interpretation of results.
4. Determination of isoagglutination group.

Introduction

The clinical study of the blood histology frequently involves four types of procedure; the estimation of the numbers of the different types of blood corpuscles in a cubic millimeter of blood, a study of the morphology of the blood corpuscles, the determination of the hemoglobin content of the blood, and a study of the coagulation mechanism. In addition to these it may be desirable in special cases to determine the percentage volume of red corpuscles or the volume of the blood, or the fragility of the red corpuscles. If transfusion is to be done, the isoagglutination group of the patient and donor must be determined.

Notes.—1. An approximate estimate of the hemoglobin content of the blood and an inspection of the stained blood film should form a part of the complete clinical examination of every patient. These very simple procedures in addition to certain signs and symptoms will indicate what further studies of the blood, if any, are desirable.

2. In any case in which there is fever, or enlargement of the spleen or peripheral lymph nodes and in any case in which inspection of the stained blood film reveals an obvious increase of the white corpuscles or an abnormal preponderance of any variety of cell or the presence of immature or abortive white corpuscles, a differential count and a count of the total number of white corpuscles per cu. mm. of blood should be made.

3. If the hemoglobin content is found to be below 80 per cent. or above 120 per cent. or if inspection of the stained blood film reveals any abnormal red corpuscles a count of the red corpuscles should be made.

4. The percentage of reticulated red corpuscles should be estimated in any case of severe anemia and in any case with or without anemia in which there is jaundice and enlargement of the spleen. In the latter case, if the percentage of reticulated red cells is increased the fragility of the red corpuscles should be determined.

5. It is important, of course, to determine the coagulation time and to enumerate the platelets in any case in which there is a history of undue bleeding following trauma or in which there is a history of spontaneous hemorrhage from mucous membranes or into the skin.   Also, in any case showing jaundice and in which operation is contemplated it is usually desirable to determine the coagulation time.

6. Inasmuch as the level of the platelet count is, in some measure, an indication of the activity of the bone marrow, an estimation of the number of platelets, either by actual count or by careful inspection of stained blood films should be made in every case showing anemia or polycythemia regardless of whether pathological hemorrhage has occurred.

7. Persons exposed to the influence of Roentgen rays or radium and persons coming in contact with certain industrial poisons, especially benzene, tetrachlorethane, lead and carbon monoxide, should submit to frequent examinations of their blood even though symptoms and signs are wanting.

The Blood Histology

Counting the Corpuscles.—Principle of the Method.— Blood obtained by puncture of the skin with a sharp needle is allowed to flow into the capillary tube of a special pipette until a definite indicated volume is obtained in the capillary tube. The blood is diluted by aspirating into the pipette sufficient diluting fluid to fill the pipette which is then shaken thoroughly to ensure proper mixing.   A drop of this diluted blood is placed in a transparent glass counting chamber and the counting chamber is covered with a special glass cover slip and placed on the stage of a microscope.   After a few minutes the corpuscles will have settled and a count of the number of corpuscles seen in a definite area of the counting chamber is made.   Knowing the volume to which the blood has been diluted and the number of corpuscles counted in a given volume of the diluted mixture, it is a simple matter to calculate the number of corpuscles in one cubic millimeter of blood.

The Counting Chamber.—Various types of counting chambers are in use and these differ from one another chiefly in shape and ruling.

In the Thoma-Zeiss type, as in most counting chambers, the depth of the chamber is .1 mm. and the counting surface is
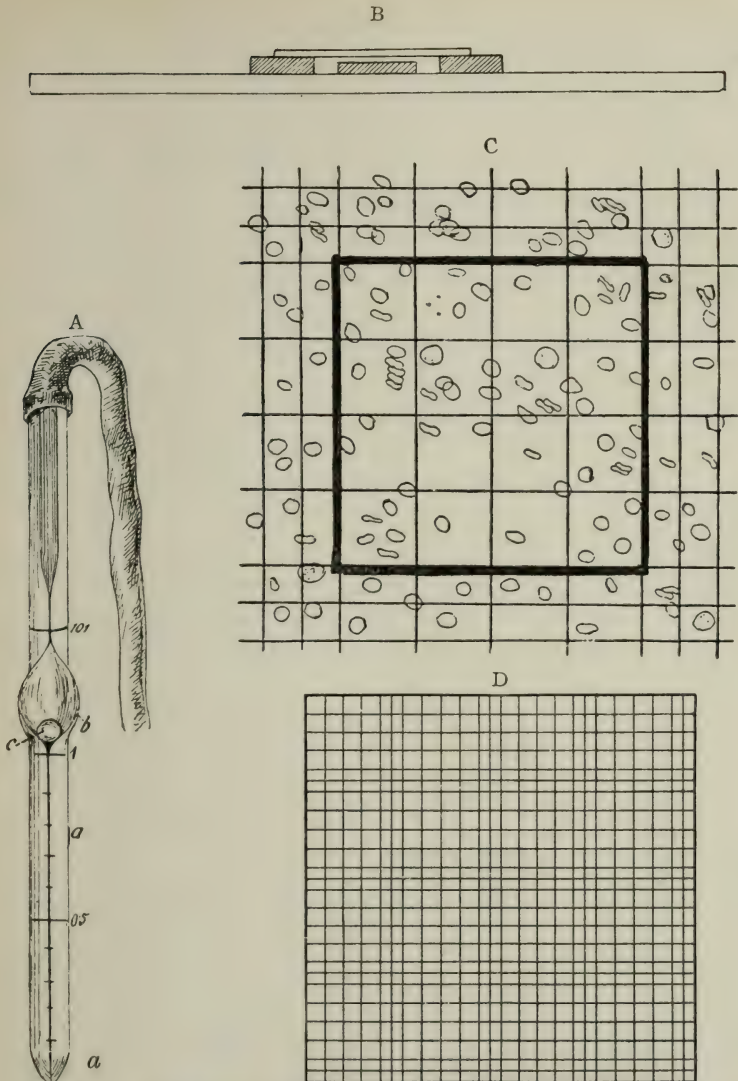
FIG. 143.—Thoma-Zeiss blood-counting apparatus (Limbeck): A, pipette for diluting blood in making count of red corpuscles; *a*, capillary tube; *b*, chamber for mixing the blood with the diluting fluid, *c*, glass pearl to aid in mixing blood with the diluting fluid. The pipette used for diluting the blood in counting the white corpuscles is of the same shape but the bulb is smaller and the graduation above the bulb is marked 11. Morever, the relation of the volume of the capillary tube to the volume of the bulb is such that a dilution of 1–10 or 1–20 can be made. B, cross-section of the chamber in which the blood corpuscles are counted. C, section of the field on which the blood corpuscles are counted showing a group of sixteen small squares each 0.0025 sq. mm. in area. D, diagram of the middle millimeter square.

divided into nine large millimeter squares.   The middle milli-
meter square is traversed by nineteen horizontal and nineteen
vertical lines which divide the square into four hundred small
squares the borders of the peripheral small squares being formed
by the borders of the millimeter square itself.   Each of
these small squares thus has an area of 0.0025 sq. mm.
Moreover, between every fourth and fifth line of the middle
square an additional line is drawn, the effect of which is to
separate the small squares into sixteen groups each containing
sixteen small squares.   Since the area of one small square is
0.0025 sq. mm., the area of a group of sixteen such squares is
16 × 0.0025 or 0.04 sq. mm.

   At the four corners of the ruled counting chamber are
to be seen four millimeter squares each divided into sixteen
small squares each 0.0625 sq. mm. in area.

   Counting the Red Corpuscles.—The type of pipette used
permits conveniently a 1:100 or 1:200 dilution of the blood,
the constriction above the bulb being marked 101.   The dilut-
ing fluid recommended is Gowers solution which has the
following composition:

Sodium sulphate,	7.5 grams;
Acetic acid,	20.0 c.c.;
Water,	120.0 "

This solution keeps indefinitely.

   Procedure.—Allow the blood to flow into the capillary tube up
to the 0.5 or 1.0 mark according to whether a 1:100 or 1:200
dilution is desired.   Touch the tip of the tongue to the mouth
piece to hold the blood at the desired level, quickly wipe off the
tip of the pipette and immerse in the diluting fluid.   Draw up
the diluting fluid to the 101 mark, wipe the tip of the pipette
and shake thoroughly for two minutes.   Both ends of the
pipette must be closed.   The mixing of the blood with the
diluting fluid is facilitated by the presence of a small glass pearl
in the bulb of the pipette.

   Now express two or three drops of the fluid from the pipette
by placing the thumb over the upper end of the pipette and

grasping the bulb in the palm of the hand.   Place the special
cover-slip over the chamber and allow the chamber to fill by
touching the tip of the pipette to the edge of the cover-slip.
If the counting chamber is clean and has been properly filled
"Newton's Rings" will be seen between the cover-glass and
the raised glass beneath.

Wait two minutes to permit the corpuscles to settle.   Then,
using a high power dry objective lens, count all the corpuscles
seen in one of the groups of sixteen small squares and record
the number counted.   Continue to count the number of corpus-
cles in these groups of small squares until at least 600 cor-
puscles have been counted.   Divide the total number corpuscles
counted by the total number of groups of squares counted.   The
result is the probable mean number of corpuscles on the surface
of one group of squares, a surface of 0.04 sq. mm.   Since the
depth of the chamber is 0.1 mm., it follows that the number
of corpuscles in 1 cu. mm. of diluted blood will be $\dfrac{10}{0.04} = 250$
times the mean number of corpuscles in one of the groups.
If this figure be multiplied by the magnitude of the dilution the
result will represent the corpuscles in 1 cu. mm. of blood.
In other words $\dfrac{\text{Corpuscles} \times \text{dilution} \times 250}{\text{No. groups of 16 squares}} = $ corpuscles in
1 cu. mm.   The results of three drops of diluted blood should
be averaged.

The mean number of red corpuscles per cu. mm. of blood
in adult males is generally taken to be 5,000,000 and in the
female, slightly less, 4,500,000.   At birth, the number is usually
somewhat greater, 6,000,000 to 6,500,000 and may even
increase during the first few days of life to 7,000,000 or more.
It then rapidly falls to a level slightly above that of the adult
from which it slowly descends during childhood.

Although as many as 6,000,000 to 6,500,000 red corpuscles
per cu. mm. of blood may rarely be observed in apparently
healthy adults, counts as high as this are usually pathological
though they do not necessarily signify disease of the blood
forming organs, erythremia.   Slight elevations in the red cell

count to 6,000,000 or 7,000,000 per cu. mm. are usually due to functional disturbances, dehydration and congenital heart disease especially in infants and young children, and in adults to myocardial incompetency and anoxemia from any cause—high altitudes, chronic emphysema, chronic illuminating-gas poisoning.

Many apparently healthy adults show occasionally a red cell count as low as 4,000,000 per cu. mm. It is frequently stated that such slightly subnormal counts are the result of a transient over dilution of the blood but this is more difficult to demonstrate as a cause of the depressed red cell count than is a transient concentration of the blood in many cases showing a slightly elevated red cell count.

A count of the red corpuscles less than 4,000,000 must be considered as pathological although, especially in women, slighter degrees of reduction in the number of the red corpuscles, 3,000,-000 to 4,000,000 per cu. mm. even though persistent at about this level frequently are not the result of a fundamental disturbance of the hemopoietic system. Although a red cell count below 3,000,000 may continue for a long time as the result of repeated blood loss, a count persistently as low as 2,000,000 or less must always arouse grave suspicion as to the presence of a permanent cause for the anemia—pernicious anemia, malignant disease, leukemia, cirrhosis of the liver, hemolytic jaundice, etc.

Counting the White Corpuscles.—The type of pipette used permits of a 1:10 or 1:20 dilution of the blood and the constriction above the bulb is marked 11. The diluting fluid recommended is a 0.3 per cent. solution of acetic acid. This fluid permits laking of the red corpuscles so that the only corpuscles readily seen are the leucocytes. The blood is collected, diluted and placed in the counting chamber precisely as it is in the case of the red corpuscles. The count is made with a low power dry objective and all the leucocytes in 1 sq. mm. are counted. This number multiplied by 100 or 200 according to the magnitude of the dilution gives the number of leucocytes in 1 cu. mm. of blood. The results of three drops of diluted blood should be averaged.

The total number of leucocytes per cu. mm. of blood varies slightly in different normal individuals and from time to time in the same individual. In normal individuals, under ordinary circumstances, however, the count of the white cells rarely falls below 5000 or exceeds 12,000 per cu. mm. of blood.

Counting of the Blood Platelets.—Method of Wright and Kinnicut.[1] Wright and Kinnicut's description of their method of counting the blood platelets follows.

The blood is mixed with a diluting fluid, in the proportion of 1 to 100, by means of the pipette used for counting red blood corpuscles, and the counting is done in the ordinary blood-counting chamber with a high-power dry objective. The diluting fluid consists of three parts of an aqueous solution of potassium cyanide (1:1400) and two parts of an aqueous solution of brilliant cresyl-blue (1:300). These two solutions must be kept in separate bottles, and mixed and filtered immediately before withdrawing the sample for counting. After the counting chamber is filled it is left at rest for ten or fifteen minutes in order that the blood-platelets may all settle to the bottom of the chamber and be more easily and accurately counted.

The platelets appear as sharply outlined, round or oval or elongated, lilac-colored bodies, some of which form a part of a small sphere or globule of hyaline unstained substance. The red cells are decolorized and appear only as "shadows," so that they do not obscure the platelets. The nuclei of the white cells are stained a dark blue, the cytoplasm light blue. If the technique is correct, there should be no precipitate in the preparation.

The cresyl-blue solution is permanent, but should be kept on ice in order to prevent the growth of yeasts. The cyanide solution should be made up at least every ten days. It is, of course, necessary that the solution be made from pure potassium cyanide which has not undergone decomposition. As already stated, the two solutions must be mixed and filtered immediately before using, because after filtration, if the mixture is allowed to remain exposed to the air for a short time, a precipitate will form in it. After the diluting fluid has been mixed

with the blood in the pipette, however, no precipitate forms and, as the platelets do not quickly break up in the mixture, the counting may be done after some hours, if necessary. For example, a count immediately after filling the pipette was 258,000, and another count from the same filling of the pipette, made eighteen hours later, was 253,000.

A proper technique yields a remarkably even distribution of the platelets in the chamber. For all practical purposes the counting of the platelets in 100 small squares is sufficient, but for greater accuracy all 400 small squares should be counted, or 200 small squares in each of two fillings of the chamber.

The total number of platelets per cu. mm. of blood varies in normal adults from about 225,000 to about 400,000. In normal infants and children, the count may be much higher, 600,000.

Notes.—1. The most convenient parts of the skin for obtaining blood are the lobe of an ear, the extensor surface of a finger just above the nail, the heel and the bottom of a toe. The lobe of the ear has the advantage that the surface is sharply convex which facilitates obtaining blood for preparing films. Whatever part is selected must be very gently massaged, washed with 70 per cent. alcohol and dried with filter paper. Much manipulation may cause extravasation of fluid which might dilute the blood and is therefore to be avoided.

2. The skin should be punctured with a sharp, sterile Hagedorn needle. The thrust should be deliberate and quick and the tip of the needle should penetrate 3–4 mm. beneath the surface of the skin in order to ensure a reasonably rapid flow of blood. The first drop of blood that appears should be wiped away with clean gauze.

3. The tip of the pipette should be brought in contact with the drop of blood as soon as it appears. This is especially important in obtaining blood for counting the platelets. Delay permits coagulation of the blood which vitiates the count. For the same reason it is important that the blood be brought in contact with the diluting fluid without delay.

4. It will be found convenient to use a rubber connecting tubing about 12 inches long. A shorter length makes the reading of the graduations on the pipette very awkward.

5. In the older types of counting chamber the ruled glass plate was cemented to the large glass slide and this necessitated great caution in cleaning inasmuch as the cement was soluble in alcohol, ether, and chloroform and was melted by heat. Recently, the Bausch and Lomb Optical

Co. has placed on the market a counting chamber in which the ruled plate is welded to the glass slide.

6. In the circular type of counting chamber the ruled surface is completely surrounded by a moat which separates the depressed ruled surface from the edges of the slide. In order to fill such a chamber it is necessary first to place a drop of the diluted blood on the ruled surface and then cover with the special cover slip. In the Levy-Neubauer type of chamber the ruled glass plate is oblong and the depressed surface extends to the edges of the slide, only two sides of the ruled plate being bordered by moats. In this counting chamber it is thus possible first to apply the cover slip and then fill the counting chamber by touching the tip of the pipette to the edge of the slide and allowing the diluted blood to pass between the cover slip and the depressed plate, an act accomplished by capillary attraction.

7. If accurate counts are to be made it is necessary to compare the counting chamber and pipettes with a standardized counting chamber and standardized pipettes such as the ones certified by the United States Bureau of Standards.[1]

8. Unless the phenomenon of Newton's rings is observed one is not sure that particles of dust are not present or that the counting chamber is not overfilled with fluid so that the depth of the fluid is greater than 0.1 mm.

9. It will be observed that the diluting pipettes are marked just above the bulb by the number 11 or 101 as the case may be. This is because the diluting fluid in the lower capillary of the pipette does not mix with the blood. That is, the volume of the pipette is 11 or 101 units but the actual volume of diluted blood is only 10 or 100 units. It is therefore necessary to express several drops of fluid from the pipette before putting any in the counting chamber.

9. It is essential that the counting chamber and cover-glass be absolutely clean and dry and that there be no air bubbles in the fluid in the counting chamber. After use the cover-glass and the counting chamber should be rinsed with cold water and wiped with chamois skin or clean linen.

10. In order to avoid errors in counting the corpuscles it is customary to observe the following rules:

(1) Always begin the count at the uppermost left hand square of a group of squares and count the corpuscles from above downwards.

(2) Count all the corpuscles which touch the upper and left hand borders of a square together with all the corpuscles in the square. Do not count corpuscles touching the lower and right hand borders of a square.

11. Before beginning to count the corpuscles it is well to examine the entire field with a low power objective lens in order to make sure that there is an even distribution of the corpuscles. Uneven distribution may be caused by dust or by insufficient shaking of the pipette. More errors in

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counting blood corpuscles can be traced to insufficient mixing of the blood with the diluting fluid than to any other cause.

12. After use the rubber tubing should be attached to the capillary end of the pipette and the contents forced out. The pipette should then be washed with three washings of water, three of absolute alcohol and one of ether. Air should be sucked through after washing with ether in order to promote drying.

13. If any coagulated albumin remains attached to the sides of the pipette it may be removed by filling the pipette with a solution, recommended by Wright, and heating at 37° C. for several hours. The solution has the following composition:

Sodium carbonate,	5.0 grams;
Pancreatin,	0.5 "
Chloroform,	0.5 c.c;
Water,	1000.0 "

If pancreatin is not available, the pipette should be filled with a solution of ammonia, made by dissolving 4 c.c. of the concentrated solution in a liter of water, and letting stand over night. It should then be filled with concentrated nitric acid, allowed to stand several hours, and then washed in the usual manner. If the tip of the pipette is clogged by coagulated albumin it may be possible to remove the clot by means of a stiff horse-hair but in no case should wire be used.

Counting All Types of Blood Corpuscles with the Use of a Single Diluting Fluid.—Method of Buckman and Hallisey.[2] The diluting fluid recommended is Edward's modification of Buckman and Hallisey's fluid and is prepared as follows.

Eight grams of cane sugar and four-tenths of a gram of sodium citrate are dissolved in 100 c.c. of distilled water. Fifty milligrams of brilliant cresyl blue are added to this solution, the mixture shaken for five minutes and then filtered once through three thicknesses of fine mesh filter paper.

Blood is obtained, diluted and transferred to the counting chamber precisely as it is in the method for counting red corpuscles described above.

When two minutes have elapsed after placing the diluted blood in the counting chamber the red corpuscles are counted precisely as detailed above. After the red corpuscles have been counted the white corpuscles may be counted using a low power dry objective and counting all the white corpuscles in 8 of the 9

sq. mm. The result multiplied by $\frac{10}{8} \times 200 = 250$ will, of course, give the number of white corpuscles per cu. mm. of blood. After the white corpuscles have been counted, or after a lapse of ten minutes, the platelets will have settled and can then be counted. Using a high-power dry objective lens the number of platelets seen in two of the corner square millimeters is counted. Since the dilution of the blood is 1 : 200, the number of platelets seen in 2 sq. mm. multiplied by 1000 gives the number of platelets in 1 cu. mm. of blood.

The results of three drops of diluted blood should be averaged.

Notes.—1. No preservative should be added to this diluting fluid inasmuch as this will cause clumping of the platelets. If placed in the ice-chest the fluid will keep indefinitely.

2. Diluted blood will be satisfactorily preserved in the pipette for twelve hours after obtaining the specimen.

3. In diluting the blood for the purpose of counting the platelets the object sought is the bringing of the diluting fluid in contact with the blood in an unaltered state. When blood is obtained from the capillaries through the skin some admixture of tissue juice is inevitable and this permits agglutination of the platelets and leads to a count that is too low. This difficulty may be overcome by the use of a paraffin-lined miniature transfusion tube with a curved outlet tube ground to fit a hypodermic needle. The needle with tube attached is inserted into a vein and the pipette is dipped into the blood as it wells up in the tube. Experience has shown that platelet counts made on blood so obtained are from 10 to 15 per cent. higher than when made on blood from the same individual at the same time but obtained from capillaries. In clinical work, however, this difference is of no significance.

4. The presence of particulate material in the counting chamber from whatever source derived and regardless of the kind of diluting fluid used, permits clumping of the platelets. Hence any specimen containing precipitated stain, incompletely hemolyzed red corpuscles, dust, etc., must be discarded.

Technique of Estimation of the Percentage of Reticulated Red Corpuscles.—Significance of Reticulated Cells.—By means of an applicator the tip of which has been dipped in a 1 per cent. aqueous solution of egg-albumin and wiped off, transfer to a clean slide a drop of 0.3 per cent. aqueous solution of brilliant cresyl blue. Spread the drop on the slide by moving the tip of

the applicator against the slide in a circular motion for about one minute. Then allow to dry. Prepare a dozen or more of these slides at a time.

Obtain a small drop of blood on the central part of a clean cover slip and quickly invert over a drop of brilliant cresyl blue solution which has been previously allowed to dry on a glass slide.

Examine with oil immersion lens. The field should be of a greenish blue color and there should be no sediment of any kind. The reticulated cells will be readily made out as well as stippled cells and polychromatophilic cells. Hemoglobin will be stained a light greenish blue and the reticulum and nuclear fragments a deep night blue. Count as reticulated cells only those which show a definite skein. Polychromatophilic and stippled cells are also young cells but should not be included among the reticulated cells unless they show a definite skein. The counting of the reticulated cells is best accomplished by placing a ruled micrometer disc or a diaphragm perforated with a pin-hole between the two lenses of the ocular system, and then causing the red cells to pass under this opening noting the number of skeined cells seen in counting one thousand red corpuscles.

Notes.—1. If the cover slip and slide are clean the blood will spread quickly and evenly between the two.

2. The preparation should be examined at once. After the lapse of an hour the red cells are apt to show crenation and may even become hemolyzed.

3. In this same preparation a rough but for ordinary clinical purpose a sufficiently accurate estimate of the number of platelets can be obtained. The platelets will be stained lilac and should not be clumped. Using a number 5 X ocular system and 1.25 N.A. oil immersion one will see in normal blood about 10 to 20 platelets per field. Assuming that the spread is uniform. and this is fundamental to accuracy, one would record that the platelets were slightly to moderately increased if 20 to 30 per field were seen, over that, markedly increased, under 10 but over 5 definitely but slightly diminished, under 5, markedly diminished. It is useless to attempt to estimate the number of platelets more accurately by this method. One therefore expresses results as follows: Normal, slightly but definitely increased, markedly increased, slightly but definitely diminished markedly diminished, extremely rare (less than one per field).

4. In case it is desired to prepare a permanent mount the following procedure described by Hawes[3] and by Cunningham[4] should be employed. After allowing the blood to be in contact with the cresyl blue for about twenty seconds smartly pull off the cover slip taking care to move it in the same plane in which the slide is held. Let dry. Then stain with Wright's stain in the ordinary method. The preparation will keep indefinitely depending on the quality of the Wright's stain.

5. In studying preparations which have been stained both with brilliant cresyl blue and Wright's stain extreme caution must be exercised in studying the leucocytes. Granules appear in the cytoplasm of the leucocytes which are not seen when the preparation is stained only with Wright's stain and these granules cause confusion in identifying cells. It is better to do differential counts on preparations stained only with Wright's stain.

Just what the reticulum seen in certain red cells stained with brilliant cresyl blue is has not been definitely determined. Certainly it does not consist of nuclear fragments. It is probable that the reticular substance represents an apparatus used by the cell during the stage between the breaking up of the nucleus and the stage of maturity. Thus we find reticular substance formed before there is any evidence of nuclear fragmentation and again we see reticulum after all evidence of stippling has disappeared. At all events the non-nucleated reticulated cell is a young cell though whether it represents an earlier stage than the polychromatophilic or stippled cell is difficult to determine. (See the article by Key.[5])

In the blood of normal adults the percentage of reticulated red cells varies from 0.6 per cent. to 1.2 per cent. Following the loss of considerable blood the percentage of reticulated cells rises and in a few days the number may reach 20 per cent. or more (marrow stimulation). On the other hand the percentage of reticulated red cells may not be abnormal in the milder degrees of anemia caused by repeated loss of small quantities of blood. A marked increase in the percentage of reticulated red cells, perhaps as high as 40 per cent., is a feature of hemolytic jaundice. A moderate increase in the percentage of these cells is often seen in pernicious anemia, usually but not always, at the onset of a remission. The reticulated red cells are usually not increased in numbers in erythemia.

A diminution in the percentage of reticulated cells is a characteristic of the aplastic anemias, primary, toxic or replacement. It is said that the percentage of reticulated cells is also much diminished in the later stages of typhoid fever and in the chronic fixed anemias of elderly adults. (For a discussion of the mechanism of the delivery of the red cells from the marrow to the blood see the monograph of Drinker et al.[6])

The Blood Histology (Continued)

Morphology of the Corpuscles.—**Preparation of the Spread**.—The best preparations are undoubtedly made with cover-slips. Blood films made on glass slides by smearing out the blood by means of another slide often have the appearance of a more nearly uniform spread than those made on cover-slips. However, the cells are apt to be fragmented in slide preparations and what is more important the leucocytes and platelets are apt to be clumped at the ends of the preparation and thus there is left the impression of a paucity of these elements in the middle portions of the spread. Moreover, an uneven distribution of the different forms of leucocytes results and this vitiates the differential count. The following procedure should be adopted in making cover slip preparations.

Collect on one cover-slip a relatively small drop of blood and immediately place another cover-slip over this in such a manner that the corners of the cover-slips can be grasped conveniently by the fingers. This operation should not consume over ten seconds. Now separate the cover-slips by pulling them apart smartly and in the same plane. If protected from dust these preparations may be put aside and stained when it is convenient; though it is better not to wait longer than twenty-four hours.

The Stain.—The blood stain most generally used in America is Wright's modification of Leishman's stain. Wright's directions for preparing his stain are as follows:

To a 0.5 per cent. aqueous solution of sodium bicarbonate add methylene-blue (B.X. or "medicinally pure") in the proportion of 1 gm. of the dye to each 100 c.c. of the solution.

Heat the mixture in a steam sterilizer at 100° C. for one full hour, counting the time after the sterilizer has become thoroughly heated. The mixture is to be contained in a flask, or flasks, of such size and shape that it forms a layer not more than 6 cm. deep. After heating, the mixture is allowed to cool, placing the flask in cold water if desired, and is then filtered to remove the precipitate which has formed in it. It should, when cold, have a deep purple red color when viewed in a thin layer by transmitted yellowish artificial light. It does not show this color while it is warm.

To each 100 c.c. of the filtered mixture add 500 c.c. of a 0.1 per cent. aqueous solution of "yellowish, water-soluble" eosin and mix thoroughly. Collect on a filter the abundant precipitate which immediately appears. When the precipitate is dry, dissolve it in methylic alcohol (Merck's "reagent") in the proportion of 0.1 gm. to 60 c.c. of the alcohol. In order to facilitate solution the precipitate is to be rubbed up with alcohol in a porcelain dish or mortar with a spatula or pestle.

This alcoholic solution of the precipitate is the staining fluid. It should be kept in a well-stoppered bottle because of the volatility of the alcohol. If it becomes too concentrated by evaporation and thus stains too deeply, or forms a precipitate on the blood smear, the addition of a suitable quantity of methylic alcohol will quickly correct such faults. It does not undergo any other spontaneous change than that of concentration by evaporation.

A most important fault met with in the working of some samples of this fluid is that it fails to stain the red blood-corpuscles a yellow or orange color, but stains them a blue color which cannot readily be removed by washing with water. This fault is due to a defect in the specimen of eosin employed. It can be eliminated by using a proper "yellowish, water-soluble" eosin.

Method of Staining.—The following procedure should be adopted in staining blood films with Wright's stain:

(a) Place the cover-slip to be stained on top of a cork stopper or clasp it with a cover-slip holder. The cover-slips must be

absolutely level, otherwise some of the stain will be precipitated on the under surface.

(*b*) Cover the preparation with 10 drops of the alcoholic stain and let stand approximately one minute. Then add distilled water drop by drop until 10 drops of water have been added. Allow to stand one to two minutes.

(*c*) Pour off the stain and wash with distilled water until the thin portions of the stained film are pink.

(*d*) Blot with filter paper of fine mesh always moving fingers in the same direction, and then hold with the fingers for a moment over a gas flame.

(*e*) Mount in balsam.

Notes.—1. In order to obtain satisfactory mounts with cover-slips and slides it is essential that these be absolutely clean. New slides and cover-slips should be cleaned first with alcohol and water. They should be wiped with a piece of clean dry gauze and kept under cover. It will be found convenient to prepare a number of slides and cover-slips in anticipation of future use.

2. It is hardly worth while to attempt to clean used cover-slips. Used slides should be allowed to stand for several days covered with a solution containing 4 c.c. of strong ammonia solution in one liter of water and then washed with soapy water.

3. The drop of blood used for the spread should be small and no delay should be permitted in transferring the drop from the skin to the glass.

4. In obtaining specimens of blood it is customary to use the first few drops for counting the corpuscles and estimating the hemoglobin. After specimens for these purposes have been obtained the blood frequently ceases to flow owing to the formation of a clot. If now the part be gently massaged and the clot removed with a piece of cloth moistened with alcohol the flow will be reestablished and a second puncture of the skin will not be necessary. The first drop should be wiped off with filter paper and subsequent ones used for making spreads.

5. If the cover-slips are clean the blood will spread evenly between them. The cover-slips must be separated as soon as the film has spread and must be pulled apart in the same plane.

6. It is better to make several cover-slip spreads at a time and to examine these under a microscope before staining. Two or three good preparations should be selected for staining.

7. It happens sometimes that better results are obtained by allowing the alcoholic stain to remain in contact with the blood film longer than one

minute or by adding more than an equal amount of water or by allowing a longer time to elapse after adding the water. Under such circumstances it is desirable to standardize the time relations and the quantities of stain and water to be used for each new lot of stain.

8. In staining blood films for examination for hematozoa it is desirable that the film be treated with the alcoholic solution of the dye and water twice as long as is necessary in staining blood films for ordinary examination.

9. McNeal's stain.[8] McNeal has recently shown that the essential dyes of the Romanowsky stain are four in number rather than three: eosin, methylene blue, methylene azure and methylene violet. McNeal's tetrachrome blood stain may be obtained in powdered form ready to be dissolved in acetone free methyl alcohol in the proportion of 3 gm. of the powdered stain to 1 liter of alcohol. Or, the stain may be prepared directly from the pure dyes by mixing in the following proportions:

Water-soluble eosin,	1.0 gram;
Medicinally pure methylene blue,	1.0 "
Methylene azure, recrystallized,	0.6 "
Methylene violet, recrystallized,	0.2 "
Pure methyl alcohol,	1000.0 c.c.

The advantages of this stain are the low cost and the uniformity of results obtained. (The dry ingredients may be obtained from the Calco Chemical Company, 136 Liberty St., New York, or from the National Aniline and Chemical Company, 40 Rector St., New York.)

10. It is useless to study poorly stained blood films. Not only may time be lost but erroneous observations may be made. The preparation should show a uniform distribution of cells. There should be no precipitate of the dye. The red cells should be tinted orange or pink, the nuclei of cells should be deep blue and the platelets should be stained lilac. Specimens which show an undue preponderance of pink or blue should be discarded.

Perfectly stained preparations are rare, however, and it often happens that a specimen which is otherwise satisfactory is slightly bluer or pinker than desirable. This should be noted and the influence this will have in the appearance of the cells borne in mind. For example, in properly stained preparations the cytoplasm of the myeloblast is hyaline and "deep" blue in color. If the preparation is overstained with blue, this cytoplasm is apt to be dark, navy blue in color. This may render the nucleus almost indistinguishable and thus make possible the confusion of this cell with a megakaryocyte nucleus. On the other hand, if there is a preponderance of pink the cytoplasm may be very much lighter in color and the cell might be confused with an immature lymphocyte,

11. Schultze's oxidase reaction used in differentiating lymphocytes from myelocytes is described on page 522. McJunkin's method of demonstrating phagocytosis by endothelial leucocytes is described on page 115.

Examination of the Stained Specimen.—The examination of the stained specimen of blood includes a study of the platelets, the red corpuscles and the white corpuscles and a search for parasites. The study of blood parasites is described in the section dealing with animal parasites and will not be discussed here.

Platelets.—It is impossible to obtain an estimate of the number of platelets from blood films made in the ordinary manner and stained with Wright's Stain. A marked diminution or a great excess of platelets will be apparent, however, and should be noted. Otherwise, it should be recorded that the platelets appear normal in numbers. It should be noted also whether the platelets appear normal, large or small in size, though the significance of large or small platelets is not known.

In case the total white count is in the neighborhood of 100,000 or in any case of myelogenous leukemia, a careful search for megakaryocyte nuclei should be made (Minot).

Red Corpuscles.—Note whether the *mean* of the *sizes* of all the cells is normal, greater than normal or less than normal. Large cells, macrocytes, preponderate in classical pernicious anemia and sometimes in myelogenous leukemia, in certain types of anemia associated with enlarged spleen and perhaps in erythremia. Small cells preponderate in anemia due to repeated loss of blood and sometimes in hemolytic jaundice and perhaps in other conditions. Note whether the cells *vary* in size (anisocytosis).

Note whether the cells are generally well filled with hemoglobin or whether there is achromia. Do not, of course, judge achromia by the color of the cells but rather by the width of the band of cytoplasm. This is one of the most important observations that can be made about the red corpuscles. Achromia is most marked in the condition known as chlorosis, rarely seen now. It is a feature of the anemia due to loss of blood. It is

rarely seen in pernicious anemia, aplastic anemia and in the anemia of leukemias.

Note especially the following types of abnormally shaped cells (poikilocytes):

(a) Large oval cells well filled with hemoglobin. These are said to be a feature of pernicious anemia.

(b) Cells whose *general* shape is distorted but which do not appear fragmented and which do not show pseudopod or tail-like processes: sausage-shaped cells, pear-shaped cells, flask-shaped cells, pessary-shaped cells. Such cells are common in reversible anemias.

(c) Cells which show evidence of fragmentation and which show tail-like or pseudopod-like processes; also very small cells called microcytes. These cells are seen especially in hemolytic anemias and are presumptive evidence of abnormal intravascular hemolysis. (For a discussion of the modes of red cell destruction the reader is referred especially to the article by Rous.[9])

The Color of the Cells.—Whereas the shape and size of the cells give information concerning abnormal blood formation or abnormal blood destruction the chromatic properties of the cells give information relative to the degree of activity of the marrow. Note especially the presence or absence of polychromasia, of fine and coarse stippling, and of variations in the depth of color of the cells.

Erythroblasts.—These are of three varieties: gigantoblasts— very large cells with blue-staining cytoplasm and a large nucleus staining intensely blue; megaloblasts—large cells with cytoplasm that may be blue, purplish or pink and with a large nucleus, deeply blue, single or multiple; normoblasts—small cells with pink cytoplasm and a round deep blue nucleus. Note the number of erythroblasts seen in counting 200 leucocytes. Note the number of gigantoblasts seen and whether the megaloblasts or normoblasts preponderate.

In general, the evolution of the red blood corpuscle is as follows. The first definitely recognizable form after the primitive mesoblast or hematoblast is the gigantoblast. Then

follow in succession the megaloblast, the normoblast, the reticulated red cell, the coarsely stippled cell, the finely stippled cell, the polychromatophilc cell, the adult red cell. The polychromatophilic cell is really a very finely stippled cell. Stippling and polychromasia represent merely different stages in the process of karyorrhexis. In a like manner, Howell-Jolly bodies, and Cabot rings presumably represent types of nuclear degeneration and probably have no special significance.

It must be remembered that the presence of immature red cells in the peripheral circulation does not necessarily mean increased activity on the part of the marrow. It may mean equally well a lowering of the marrow threshold by virtue of which the marrow allows immature cells to enter the circulation. Whether one is dealing with increased activity or lowered threshold can be determined only by circumstantial evidence.

The White Corpuscles.—It is impossible to determine even roughly the number of white cells by inspection of the stained blood film. A total white count of 30,000, say, will show many more cells than one of 5000 but one is not safe in making inferences from blood films as to the degree of leucopenia or leucocytosis.

It is impossible to make a differential count of the leucocytes in poorly stained specimens. One must always bear in mind the effect of a preponderance of blue or red on the appearance of the different forms of leucocytes and interpret the cells accordingly. Two hundred cells should be counted in making routine examinations. It is sometimes desirable (leukemia) to count more than 200 cells and it occasionally happens that the counting of as many as 200 cells consumes an amount of time incommensurate with the value of the information (aplastic anemia). However, differential counts based on counting fewer than 100 cells are worthless. In any case the number of leucocytes counted should be stated.

In classifying the leucocytes the plan suggested below may be followed. If cells are encountered which defy classification a schematic drawing of such cells should be made and such indeterminate cells included in the count.

In all cases showing leucocytosis or leucopenia the absolute numbers of the various types of cells as well as percentages should be recorded. Finally one should note whether there is an unusual degree of fragmentation of the leucocytes and record somewhat as follows: normal fragmentation (10 per cent. or less); marked fragmentation (10–40 per cent.); extreme fragmentation (over 40 per cent.).

Classification of the Leucocytes.—The white corpuscles or leucocytes are usually classified according to their morphology and staining characteristics. Three main groups are recognized, the myelogenous leucocytes, the endothelial leucocytes and the lymphocytes. Although the following method of classification has been found useful no claim of finality is set forth for it. The descriptions refer to blood films stained with Wright's stain. (For a discussion of the origin of the cells of the blood the reader should consult the recent article by Sabin.[10])

(a) *Myelogenous Leucocytes.*—Three distinct types, in order of maturity, are readily made out—the myeloblast, the myelocyte, and the mature, small, polymorphonuclear cell. If one bears in mind these three definite types the recognition of other cells of the series as transitional forms becomes simple.

The myeloblast is seen as a large cell, usually round, sometimes ovoid. Its cytoplasm is deep blue in color and entirely homogeneous. The round or oval nucleus is relatively large, never indented, very distinct, very dense and very deeply stained, blue or purple.

The myelocyte is usually slightly smaller than the myeloblast. Its cytoplasm is pale blue in color and contains many lilac or purple granules. The nucleus is round or oval, less dense than the nucleus of the myeloblast and often granular in structure. In contrast to the nucleus of the myeloblast, the nucleus of the myelocytes is often indistinct and may show an irregular, but never deeply indented border.

One recognizes a transitional form between the myeloblast and the myelocyte, the so-called premyelocyte or young myelocyte. According to the stage of development this cell may approximate more nearly the myeloblast or the myelocyte.

It is frequently larger than either and its cytoplasm contains few to many red or purple granules.

As the myelocyte becomes more mature certain characteristic changes occur. The cell becomes smaller, the nucleus becomes indented and the granules of the cytoplasm show distinctive staining properties. These more mature cells are designated as metamyelocytes, or as young or old polymorphonuclear cells according to their sizes and the degree of lobulation of their nuclei. They are also designated eosinophiles, basophiles or neutrophiles according to whether their granules are stained respectively red, blue or lilac. One thus refers to metamyelocytic or young polymorphonuclear or old polymorphonuclear eosinophiles, basophiles or neutrophiles.

The old polymorphonuclear neutrophile is perhaps only one-fourth the size of the myeloblast. The cytoplasm is very pale blue and shows many very fine lilac granules. The nucleus possesses four or more lobes connected to one another by very fine strands of protoplasm. The adult eosinophile like the adult neutrophile shows a multilobular nucleus. Usually it is a larger cell than the neutrophile and its cytoplasm is packed with coarse, red, refractile granules. Occasionally these granules are fewer in number, fine and pink rather than red, and under such circumstances the eosinophile may closely simulate the neutrophile. The basophile or so-called mast cell, probably a degenerated eosinophile, is the most easily recognized of all the cells in the blood. Its nucleus is multilobular and its cytoplasm is filled with granules, very large and of a very deep blue color.

In the peripheral blood of normal resting adults myelogenous cells younger than the young polymorphonuclear cells are exceedingly rare. Myelocytes and younger myelogenous cells are classically seen in the peripheral blood in large numbers in myelogenous leukemia. They are seen *often* in small numbers in erythremia, after hemorrhage, in pernicious anemia, and *rarely* in other conditions, notably, lobar pneumonia, malignant endocarditis, acute yellow atrophy of the liver and mycosis fungoides.

Whereas, in adults, the presence of many undifferentiated myelogenous cells in the peripheral blood leads one to suspect the presence of myelogenous leukemia, they may appear on slight provocation in the peripheral blood of infants and young children. Myelocytes and even myeloblasts are frequently seen in considerable numbers in the blood of infants and children suffering from conditions giving rise to a marked leuco- cytosis, notably in pneumonia and in infantile splenic anemia (von Jaksch's anemia).

(*b*) *Endothelial Leucocytes.*—These cells, like the lympho- cytes, are, in a sense, accidental cells of the blood. They differ distinctly from the other formed elements in origin, structure and function. They are derived from the endothe- lium lining the capillaries and small veins and also from that lining the lymphatics and lymph sinuses. They are large cells, the macrophages in contrast to the microphages or polymor- phonuclear neutrophiles and are especially active in englobing particles of calcium, carbon and hemoglobin, a fact which serves to differentiate them.

Two stages in the development of these cells are recognized, the early endothelial leucocyte, so-called large mononuclear cell, and the late endothelial leucocyte, so called transitional cell. The former cell has a large round or oval nucleus, often eccen- tric, surrounded by much pale blue cytoplasm in which are to be seen numerous fine to medium sized pale blue granules. In cases of malignant endocarditis endothelial cells containing granules of ingested hemoglobin rarely may be distinguished, the Herzfehlerzellen of the Germans.

As in the case of the myelogenous cells the older endothelial cells are smaller than the early endothelial cells and show indentation and even lobulation of the nucleus. The nucleus is characteristically kidney shaped or U-shaped but may show numerous lobes connected by constricted necks of nuclear material. These last may simulate polymorphonuclear neutro- philes but the resemblance is only superficial. The polymor- phonuclear endothelial cells may rarely show a few star shaped magenta granules in its cytoplasm. Otherwise, the granules

are always bluish, never lilac or pink. Moreover the proportions of the cell are larger, than those of the polymorphonuclear neutrophiles. The blood of normal individuals contains both early and late endothelial cells.

Lymphocytes.—The classical lymphocyte is a basophilic cell containing a round, dense, nucleus surrounded by a varying amount of very delicate, pale blue hyaline cytoplasm. Except in the case of very young cells, the lymphocytes contain no granules. These cells phagocyte nothing.

Young lymphocytes, rarely seen in the peripheral blood of normal individuals but seen in large numbers in lymphatic leukemia, are large cells about the size of a myeloblast. The nucleus is relatively small, very dense and may contain one or more nucleoli. It is often eccentrically placed and may be slightly indented. The cytoplasm is abundant, fragile and often frayed at the edges. It may contain a few star shaped magenta granules.

As the lymphocyte matures its cytoplasm shrinks. One distinguishes in the peripheral blood of normal individual the large lymphocyte and the small lymphocyte. These differ from one another almost wholly in respect to the quantity of cytoplasm surrounding the nucleus. But occasionally the large lymphocyte contains a very few pinkish granules. The small lymphocyte is the smallest variety of leucocytes. It appears as a dense nuclear disc, not much larger than a red corpuscle, surrounded by an exceedingly thin rim of pale blue hyaline cytoplasm.

Abortive Cells.—The cells hitherto described may be called normal cells although not all of them appear in the blood of normal individuals. In addition to these one encounters in various pathological processes, notably in the leukemias, many cells which do not conform to type. These are best looked upon as abortive or sport cells. Abortive cells are never seen in normal blood. A few frequently occurring types of abortive cells may be mentioned.

(a) *Lymphocyte Series.*—Turk irritation forms. Pathological lymphocytes. These cells bear a superficial resemblance to

large lymphocytes. They are frequently larger than large lymphocytes. In shape they are sometimes round, often oval and often very much elongated. Their cytoplasm is of a dull and rather deep blue color and is made up of numerous fine basophilic granules. The nucleus is usually eccentric, elongated and sometimes indented. Sometimes two nuclei are seen. The nucleus often contains nucleoli and vacuoles and may contain eosinophilic granules. These cells are seen in certain pathological processes, notably in acute inflammatory processes, in serum sickness, during the course of reactions following injections of foreign proteins and sometimes in lymphatic leukemia and von Jaksch's anemia.

Another type of unusual lymphocyte sometimes seen in lymphatic leukemia, infantile splenic anemia and other conditions is a small, round or oval cell with a dense granular and slightly indented nucleus surrounded by a small amount of pale cytoplasm. This cytoplasm frequently contains small pinkish granules.

(b) *Myelogenous Series.*—"X-cells." Abortive cells. These are cells about the size of a myelocyte. They are round or oval with a blue cytoplasm which is usually hyaline and not granular. The nucleus is frequently lobulated, usually eccentric and contains vacuoles and nucleoli. Often the nucleus is reduced to a small polar mass of cytoplasm. In addition to these one often sees in myelogenous leukemia many bizarre cells; for example, a cell the size of a red corpuscle with a blue nucleus about the size of that seen in the normoblast but in this case usually indented. This nucleus may be surrounded by large eosinophilic granules densely packed together. Or again one sometimes sees a cell about the size of a myelocyte with a rather indistinct roundish nucleus with the cytoplasm packed with large, deep blue granules. Or again, one encounters a very large cell with a rather deep blue and hyaline cytoplasm containing a rather indistinct lobulated large nucleus, a myeloblast, as it were, with the nucleus of a meta-myelocyte. It is useless to classify these sport or abortive cells because such a classification would be without significance. These cells are readily

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recognized. When they occur in any considerable numbers, say over 5 per cent., in myelogenous leukemia, they suggest disorderly blood formations and an unfavorable outlook.

In the blood of normal adults the polymorphonuclear neutrophiles (adult and young) constitute from 60–75 per cent. of the total number of leucocytes. Endothelial leucocytes (early and late together) make up from 2–8 per cent. eosinophiles, 0–2 per cent., mast cells, 0–1 per cent., lymphocytes, 20–30 per cent.

In infants and in children under three years of age the percentage of lymphocytes is usually greater than the percentage of polymorphonuclear cells.

It must be borne in mind, however, that one often finds considerable variation from these limits in persons in whom no demonstrable pathology is present. For example, in counting 500 leucocytes one may encounter a single myelocyte in the blood of an individual who is apparently healthy. Such a finding must be interpreted as accidental and without significance.

Pathological Variations in the Leucocytes.—In considering the pathological variations from the normal absolute leucocyte count and the differential count these remarks apply to outspoken conditions in which these variations are definite, are fairly constant, and are of actual assistance clinically.

A. Leucocytosis (over 12,000 leucocytes per cu. mm. of blood).

I. Leucocytosis with increase in the polymorphonuclear neutrophiles is the rule in

(*a*) Acute suppurative processes and septicaemia due to pyogenic cocci;

(*b*) Lobar pneumonia;

(*c*) Meningitis—meningococcus, streptococcus, staphylococcus, pneumococcus, practically always—tubercle bacillus, usually—spirochaete pallidum, sometimes.

In the above conditions a total white cell count of from 14,000 to 35,000 is the rule. Within these limits, variations in the count are not of great clinical significance except that sudden rises in the count usually but not always indicate clinical extension of the process. Counts above 35,000 usually carry a

grave prognosis except that in lobar pneumonia counts of 40,000 are entirely compatible with recovery. A count of 50,000 in lobar pneumonia is definitely a bad sign. Similarly, a count of less than 10,000 in frank, lobar pneumonia is distinctly a bad sign. In all of these cases the increase in the white count is due to an absolute increase in the number of polymorphonuclear neutrophiles. Accordingly, the percentage of lymphocytes, endothelial cells, eosinophiles, and mast cells all together may be as low as from 1 per cent. to 15 per cent. depending on the total white count.

(d) Hemorrhage. Following a profuse hemorrhage (traumatic, non-pathological) a leucocytosis is the rule, usually not over 20,000 white cells per cu. mm. The blood picture in this case differs, however, from the picture in the first three instances for whereas in the former the increase in the total white cell count is due almost wholly to an increase in the mature polymorphonuclear neutrophiles, after hemorrhage young polymorphonuclear cells and even myelocytes may appear. There may be an increase in the number of endothelial cells. The lymphocytes are not increased in absolute numbers. Equally characteristic of the blood after hemorrhage are two other features: increase in the number of platelets and presence of immature red cells, often erythroblasts.

(e) Alimentary leucocytosis. A leucocytosis amounting to 12,000 to 14,000 white cells per cu. mm. may occur following a meal.

(f) Hodgkin's disease. Leucocytosis may or may not be present. When present the increase in the total number of white cells may be due exclusively to increase of the polymorphonuclear neutrophiles but is commonly due also to an increase in the number of eosinophiles and endothelial cells.

(g) Myelogenous leukemia. The total count may vary in untreated cases from normal to over a million. The increase may be due largely to adult polymorphonuclear neutrophiles but there are always present younger myelogenous cells the percentage of which varies with the severity of the process. The histology of the blood, rather than the total white count,

is the most important single laboratory diagnostic guide in this condition (Minot). Abortive or sport cells are often seen in this disease and suggest an unfavorable prognosis.

(*h*) Erythremia. In this disease, in addition to the increase in red cells and platelets, there is characteristically a leucocytosis of from 15,000 to 30,000. The increase is due to an increase in the polymorphonuclear neutrophiles. Myelocytes may be present.

(*i*) Miscellaneous conditions. In the following conditions a leucocytosis from 12,000 to 30,000 with increase in the percentage of polymorphonuclear neutrophiles may occur: Acute articular rheumatism, poliomyelo-encephalitis (early stages), acute follicular tonsillitis, infectious jaundice, scarlet fever, intestinal obstruction, myxedema, diphtheria, capillary bronchitis, bronchopneumonia, carcinoma, sarcoma, urethritis, rabies and bronchiectasis. The leucocytosis in these conditions is inconstant and not usually of clinical assistance.

II. Leucocytosis with increase in the lymphocytes as the outstanding feature.

(*a*) In the following conditions a leucocytosis due to an absolute increase in the number of lymphocytes is the rule: whooping cough, mumps, glandular fever, Graves' disease, atropine poisoning (sometimes), acute infections of the pharynx and tonsils with enlargement of the cervical lymph nodes (sometimes), lymphatic leukemia, infantile splenic anemia (von Jaksch's anemia). In the last named condition immature cells both of the lymphocyte series and the myelogenous series are frequently a feature. Frequently, also, there is a slight eosniophilia. Except in the case of lymphatic leukemia, the leucocytosis is rarely over 30,000.

III. Eosinophilia.

(*a*) Leucocytosis due entirely to an absolute increase in eosinophiles does occur (dermatitis herpetiformis, anchylostomiasis trichiniasis) but is rare. By eosinophilia is usually meant an increase in the percentage of eosinophiles above 3.

(*b*) A moderate or slight eosinophilia may occur in a variety of conditions of which the following is a partial list. In these

conditions the eosinophilia is by no means constant and its significance is not at all understood. There may or may not be a leucocytosis.

1. Condition in which there is a leucocytosis due to an absolute increase in the number of lymphocytes (see above).

2. In the convalescent stage of certain infectious diseases, notably scarlet fever, lobar pneumonia, acute articular rheumatism, chicken pox, malaria.

3. Affections of the bones, notably rickets, osteomalacia, osteosarcoma.

4. Miscellaneous conditions especially pernicious anemia, Addison's disease, ovarian disease.

5. Infections, especially osteomyelitis and gonorrhoea.

6. Drugs: pilocarpine, camphor, hydrogen sulphide.

7. Following the parenteral injection of foreign proteins.

8. Malignant disease with metastases: carcinoma and sarcoma.

(c) In the following conditions the eosinophilia is frequently marked and occurs almost always in connection with the leucocytosis.

1. Hodgkin's Disease.

2. Myelogenous Leukemia.

(d) In the following conditions the eosinophilia may be marked and may occur with or without a leucocytosis and the leucocytosis may or may not be due entirely to the eosinophilia.

1. Bronchial asthma and hay fever (cf. foreign protein).

2. Certain Skin Diseases, especially pemphigus, erythema bullosus, dermatitis herpetiformis, hydroa, herpes iris, psoriasis and eczema (rarely). (cf. foreign protein).

3. Following splenectomy.

4. Certain diseases due to animal parasites, especially those due to the following: Anchylostoma duodenale and Necatur Americanus, Taenia solium and saginata, Dibothriocephalus latus, Bilharzia hematobia, Taenia mediocanellata, Filaria sanguinis hominis, Trichinella spiralis, Echinococcus.

(e) A slight degree of eosinophilia with leucocytosis may occur but is usually absent in the following conditions: infec-

tions due to Ascarius lumbricoides, Trichocephalus dispar, Oxyuris vermicularis, Pediculi, Acarus scabiei.

IV. Mast Cells.—The only condition in which a significant increase in the mast cells occurs is in myelogenous leukemia. Certainly any percentage of mast cells greater than two is pathological, however.

V. Leucocytosis, associated with an increase of the endothelial cells. Any percentage of endothelial leucocytes over ten is abnormal.

(a) A leucocytosis due largely to an increase in the endothelial cells is seen in tetrachlorethane poisoning.

(b) A leucocytosis due principally to an increase in poly-morphonuclear neutrophiles but also associated with a relative and absolute increase in the endothelial cells and eosinophiles is a feature of Hodgkin's disease but Hodgkin's disease may occur without this picture. The polymorphonuclear neutrophilic leucocytosis in Hodgkin's disease often precedes the increase of the endothelial cells and eosinophiles.

(c) A leucocytosis due largely to increase in the lymphocytes and endothelial cells, with or without eosinophilia, and consequent suppression of polymorphonuclear neutrophiles is a feature of acute infectious mononucleosis. The same picture may be seen also in chronic infections of the sinuses of the head and the tonsils and perhaps in case of chronic infection elsewhere in the body. But in case of chronic head infection a leucopenia is rather the rule (v.i.).

(d) In malignant endocarditis a marked leucocytosis due to an absolute increase in the endothelial leucocytes may occur. In this condition one occasionally sees endothelial cells containing haemoglobin particles (Herzfehlerzellen).

VI. Leucocytosis associated with increase in all the types of leucocytes so that the differential count remains normal or nearly so. This situation may be encountered in conditions causing a diminished plasma volume such as Asiatic Cholera and dehydration in infants, and may also occur in circulatory stasis.

B. Leucopenia. (Less than 5000 white cells per cu. mm. of blood.)

I. In the following conditions a leucopenia due to a suppression of the polymorphonuclear neutrophiles is the rule unless complications such as hemorrhage, meningitis, pneumonia or acute suppuration due to pyogenic cocci are present:

(*a*) Drugs: mercury, arsenic, alcohol, ether, morphine

(*b*) Starvation.

(*c*) Benzene poisoning (Benzol poisoning).

(*d*) Lead poisoning (sometimes).

(*e*) After the parenteral injection of foreign protein.

(*f*) Aplastic anemia.

(*g*) Pernicious anemia in relapse.

(*h*) Following over-exposure to X-ray or radium.

(*i*) Malaria. Here there is relative increase in the large lymphocytes.

(*j*) Typhoid fever.

(*k*) Influenza (uncomplicated).

(*l*) Dengue.

(*m*) Cirrhosis of the spleen due to whatever cause (Banti's disease, syphilis, alcoholic cirrhosis of liver, etc.).

In the three last named conditions the leucopenia is associated with a relative increase in the small lymphocytes.

II. In the following conditions a leucopenia may be present but the blood may be normal or a leucocytosis may occur.

(*a*) Tuberculosis—all forms except tuberculous meningitis. In tuberculous meningitis a leucocytosis due to absolute increase in the number of polymorphonuclear neutrophiles is the rule.

(*b*) Measles.

(*c*) Chronic infection of the head sinuses or the tonsils and perhaps chronic infection anywhere in the body. Here one frequently sees a leucopenia or a normal white cell count and a relative increase in lymphocytes, endothelial cells and eosinophiles and a suppression of polymorphonuclear neutrophiles.

(*d*) Lymphatic leukemia, treated with X-ray or radium. C. Conditions usually exhibiting a total white cell count within normal limits but an abnormal differential count of the leucocytes.

In any of the above mentioned conditions the total number of leucocytes may be within normal limits. In the following conditions a normal leucocyte count is frequently obtained but an abnormal preponderance of a particular variety of leucocyte is found:

1. Preponderance of polymorphonuclear neutrophiles: subacute suppurative conditions due to pyogenic cocci and Hodgkin's Disease.

2. Preponderance of eosinophiles: Bronchial asthma, hay fever, infection due to intestinal animal parasites (see above); pernicious anemia; Hodgkin's disease.

3. Preponderance of Lymphocytes: Typhoid Fever, Malaria, Conditions leading to cirrhosis of the spleen, influenza, lymphatic leukemia in state of remission, pernicious anemia.

4. Presence of immature or unusual forms of leucocytes; Myelogenous leukemia after treatment with radium or Roentgen ray, aleukemic leukemia, infantile splenic anemia, acute yellow atrophy of the liver, lymphatic leukemia, allergic conditions.

THE HEMOGLOBIN CONTENT OF THE BLOOD AND ITS RELATION TO THE COUNT OF THE RED CORPUSCLES

Determination of Hemoglobin Content of the Blood. The numerous methods for the determination of the hemoglobin content of the blood vary in accuracy from the roughly approximate method of Tallquist to the methods of a very high order of accuracy such as the gasometric methods of Haldane, Y. Henderson and Van Slyke and the spectrophotometric method used by Lichtenstern, Butterfield, Williamson and others.

A satisfactory clinical method is that which makes use of a Sahli Hemometer which has been standardized by comparison of figures obtained from the Sahli apparatus with figures obtained from the gasometric determination of hemoglobin in the same sample of blood. With careful technique and by

the use of a standardized instrument it is possible to estimate the hemoglobin content of the blood with an accuracy of 2 per cent. with the Sahli method. The Sahli standard should be corrected every three months.

Estimation of the Hemoglobin Content of the Blood by the Gowers-Sahli Method.—The apparatus consists essentially of (1) a closed tube containing the standard solution—a solution of picric acid and carmine the color of which closely approximates the color of acid hematin, (2) a small test tube graduated to read in percentage of hemoglobin and (3) a special pipette for use in obtaining a definite quantity of blood. The determination is carried out as follows:

Place enough tenth normal hydrochloric acid in the clean graduated tube to reach approximately to the 10 per cent. mark. Draw up in the special pipette 20 cu. mm. of blood (20 mark). Place the tip of the pipette beneath the level of the acid in the tube and blow out the blood gently, avoiding bubbles. Wash out all the blood from the pipette by sucking up the acid and expelling the blood into the graduated tube several times. Let stand until the blood has been laked and the formation of acid hematin is complete as evinced by the formation of a clear brown solution. Then add distilled water drop by drop until the color in the graduated tube is of the same intensity as that in the standard tube. Read off the percentage of hemoglobin directly from the graduations reading the bottom of the meniscus. Be sure to shake the tube (by inversion) after addition of each drop of water.

Notes.—1. The function of the hydrochloric acid is the conversion of the hemoglobin into acid hematin.

2. It is often convenient to use several graduated tubes in as much as it is permissible to wait as long as thirty minutes before diluting the acid hematin solution and in this time several specimens may be taken.

3. If bubbles form in the acid hematin solution they should be dispelled by the addition of a drop of ether.

4. It is absolutely essential that the standard tube and the tube containing the acid-hematin solution should be evenly illuminated. The best light for this purpose is indirect daylight but electric light transmitted through blue glass can be used,

5. Values for hemoglobin obtained by use of the Sahli apparatus are subject to a larger error, 5–10 per cent. when the percentage of hemoglobin lies above 120 or below 30. In such cases it is advisable to use the gasometric method described below if accurate values are necessary.

6. Palmer[11] has devised a very accurate colorimetric method for the determination of hemoglobin in which a carbon monoxide-hemoglobin standard is used and Newcomber[12] has also introduced a colorimetric method in which the color standard consists of a piece of color glass. These methods have been critically studied by Robscheit.[13] In the Palmer method more blood than can usually be obtained conveniently from skin puncture is required and the standard must be checked frequently. In the Newcomber method a colorimeter is required.

The Sahli method, if occasionally standardized by the gasometric method, gives results sufficiently reliable for most clinical purposes and if one really wants to make an accurate determination of the hemoglobin one might as well resort to Van Slyke's gasometric method which, after all, requires no very expensive apparatus and is easy to carry out.

Van Slyke's Method for Determining Hemoglobin.— The Sahli method of determining hemoglobin may be easily standardized by comparing the values obtained in a series of determinations by this method with those obtained from the same specimens of blood using Van Slyke's gasometric method. Although an even more accurate determination of the hemoglobin content of the blood may be made by the spectrophotometric method the technique of the method is rather more difficult and time consuming than is commensurate with the value of the results for most clinical purposes.

Principle of the Method.—In the gasometric method blood obtained from the vein is completely saturated with oxygen and a known quantity is then introduced into a special pipette. The blood is laked with dilute ammonia solution, the oxygen set free by addition of potassium ferricyanide and the volume of gas liberated read off from the graduated tube of the apparatus. This volume of gas is reduced to standard conditions and multiplied by a factor in order to convert the readings to percentage of hemoglobin or grams of hemoglobin per 100 c.c. of blood.

The Apparatus.—The following is taken from Van Slyke's original description of the apparatus[14] and method.[15] The

apparatus consists of a special 50 c.c. pipette provided with a three way stopcock at the top and another at the bottom. The stem of the pipette is marked by graduations representing
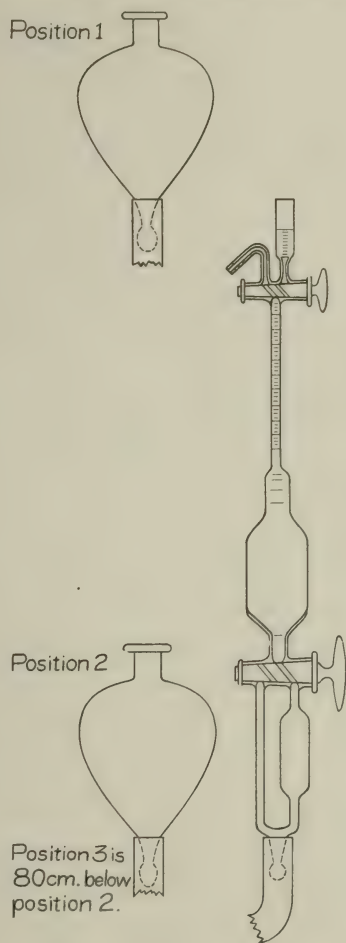
Fig. 144.—Van Slyke's gas analysis apparatus for carbon dioxid of blood, showing the three positions of the mercury bulb. The bulb is connected to the apparatus by a heavy rubber tube which is merely indicated in the drawing. The apparatus is set up on an iron ring stand (Todd, Clinical Diagnosis).

0.02 c.c. The volume of the stem and upper part of the bulb contain a volume of 2.5 c.c. represented by a mark and just below the large bulb is a graduation representing 50 c.c.

Two outlets connect with the upper stopcock e. The capillary a serves for the removal of water solution and the other capaillary connects with the cup b which receives blood in ammonia solution. The lower stopcock f connects with a tube c and a small bulb d which fuse below into a common outlet. The small bulb d serves as an outlet for the solutions after the oxygen has been liberated and through tube e mercury enters when it is desired to release the vacuum. The common lower outlet is connected with a mercury reservoir by means of heavy pressure tubing.

Before using, the apparatus must be calibrated. This is most easily done as follows: Attach a tube drawn out into a small capillary to the lower outlet of the apparatus. Immerse the end of this capillary tube into a vessel containing 75 c.c. of distilled water, turn the upper stopcock so that a connection is established between the stem and capillary a and turn the lower stopcock so that a connection is established between tube c and the large bulb. Then connect the tube a with suction and allow the apparatus to fill with water up to the stopcock e. Disconnect the suction and close stopcock f. By means of stopcock f let out water, 0.1 c.c. at a time, into a weighing bottle and weigh to an accuracy of 1 mgm.

Technique of the Determination.—The determination of the oxygen combining capacity of the blood, from which the hemoglobin content is calculated, is carried out as follows: Five drops of coprylic alcohol and 6 c.c. of ammonia solution, made by dissolving 4 c.c. of ammonia in a liter of water, are introduced into the cup and then let into the apparatus. The capillary below the cup is filled with mercury and the mercury reservoir is lowered to position 3. When the mercury level reaches the 50 c.c. mark, the lower stopcock is closed and the apparatus is then shaken for a minute. The extracted air is then expelled through the capillary leading to the cup.

About 5 c.c. of blood are obtained from a vein and transferred to a test tube containing a small amount of powdered potassium oxalate. The blood is thoroughly stirred with a glass rod and then transferred to a 500 c.c. separating funnel. The

funnel is rotated for five minutes, care being taken that the blood is spread in a thin film over the sides of the funnel.

Two cubic centimeters of air free ammonia are now forced up into the cup of the apparatus and exactly 2 c.c. of the thoroughly aerated and well stirred blood are transferred to the cup by means of a calibrated 2 c.c. pipette. The blood is now run from the cup of the apparatus into the 50 c.c. bulb, the ammonia layer following the blood and washing it in. A few additional drops of ammonia may be necessary to ensure complete removal of the blood from the cup. The apparatus is now shaken for a minute to ensure thorough mixing of blood and ammonia and is then allowed to stand five minute or until complete laking of the blood has been effected.

Four-tenths of a cubic centimeter of a saturated solution of potassium ferricyanide are now introduced into the apparatus to liberate the oxygen combined with the hemoglobin and the capillary tube leading to the cup is sealed with mercury. Oxygen is now removed from the solution by lowering the mercury reservoir until the mercury level is just above the 50 c.c. mark, closing the lower stopcock and shaking the apparatus for two minutes in a rotary motion. The solution of hemoglobin in ammonia is let into the lower bulb of the apparatus, mercury is let in through the tube c and the volume of extracted gas above the mercury is noted. The evacuation is repeated until two consecutive readings of the volume of the gas are identical.

Knowing, then, the volume of aerated blood introduced into the apparatus, the volume of oxygen and dissolved air released from this amount of blood, and the temperature and barometric pressure it is possible to calculate the quantity of oxygen reduced to standard conditions (0° C. and 760 mm.) combined in 100 c.c. of aerated blood. This is the oxygen capacity of the blood and is usually expressed in terms of volumes per cent.; c.c. of oxygen contained in 100 c.c. of blood. Assuming that 18.5 volumes per cent. oxygen correspond to 100 per cent. hemoglobin it is a simple matter to calculate the per cent. of hemoglobin of the blood specimen. The appended table

taken literally from Van Slyke's original article greatly reduces the labor of computation.

Table for computing the oxygen combining capacity and hemoglobin content of the blood.

FACTORS FOR CALCULATING RESULTS FROM ANALYSIS OF 2 C.C. OF BLOOD SATU-
RATED WITH AIR

Temperature	Air physically dissolved by 2 c.c. of blood. Subtract from gas volume read in order to obtain corrected gas volume, representing O_2 set free from hemoglobin	Factor by which corrected gas volume is multiplied in order to give:	
		Oxygen chemically bound by 100 c.c. of blood	Per cent. hemoglobin, calculated on the basis of 18.5 per cent. oxygen = 100 per cent. hemoglobin.
$C.°$	c.c.	c.c.	per cent.
15	0.037	$46.5 \times \dfrac{B}{760}$	$251 \times \dfrac{B}{760}$
16	0.036	46.3 "	250 "
17	0.036	46.0 "	249 "
18	0.035	45.8 "	247 "
19	0.035	45.6 "	246 "
20	0.034	45.4 "	245 "
21	0.033	45.1 "	244 "
22	0.033	44.9 "	242 "
23	0.032	44.7 "	241 "
24	0.032	44.4 "	240 "
25	0.031	44.2 "	239 "
26	0.030	44.0 "	237 "
27	0.030	43.7 "	236 "
28	0.029	43.5 "	235 "
29	0.029	43.3 "	234 "
30	0.028	43.1 "	233 "

Notes.—1. Before making a determination it is necessary to make sure that the apparatus is air-tight and free from gas. This is accomplished as follows: By elevating the mercury reservoir to position 1, fill with mercury the entire apparatus including the capillaries above the upper stopcock. Close this stopcock and open the lower one in such a manner that a connection is established with bulb *d*. Lower the reservoir to position 3 until the mercury reaches a level about the middle of bulb *d*. Then raise the leveling bulb to position 1. If there is no air in the appara-

tus the column of mercury will strike the upper stopcock with a sharp metallic click. If there is any gas in the apparatus this click will not be heard because of the cushion effect of the gas bubble on top of the mercury. It may be necessary to evacuate the apparatus several times before all the air in the apparatus is expelled. When the apparatus has once been rendered air-tight and air-free it is possible to keep it so indefinitely.

2. The great weight of the apparatus when filled with mercury necessitates special support. This is provided for (1) by means of an iron rod passing under the lower stopcock and (2) by means of a burette clamp which clasps the upper part of the large bulb and steadies the apparatus.

3. The stopcocks must be held in place by means of heavy elastic bands or wire springs. Otherwise the weight of the mercury is apt to cause them to be dislodged at a critical moment.

4. Ordinary vaseline or desiccator grease will not suffice for greasing the stopcock bearings. The following mixture is recommended:

Vaseline,	12 grams;
Paraffin,	1 "
Pure gum rubber,	9 "

These ingredients should be heated together on a sand bath until, with constant stirring, they are thoroughly mixed.

5. Fifty milligrams of potassium oxalate are sufficient to prevent 5 c.c. of blood from coagulating. In place of the powdered potassium oxalate one drop of a 30 per cent. solution of sodium citrate may be used. The error caused by dilution in this case is about 1 per cent.

6. It is important that the tip of the calibrated pipette used to transfer the blood from the separatory funnel to the cup of the apparatus should reach to the bottom of the cup. For this purpose the lower delivery mark should be 2–3 cm. above the tip.

7. It is impossible to transfer all of the solution of laked blood from the main bulb of the apparatus to bulb d. A small column of this solution remains on top of the mercury after the vacuum has been released. One reads the volume of gas above the water meniscus of course, but the leveling bulb is brought to the level of the mercury meniscus (theoretically to a level about $\frac{1}{13}$ of the height of the water column above the mercury meniscus).

8. Modifications of this apparatus and technique more recently described by Van Slyke, as well as methods described by others, permit even more accurate determinations of the oxygen combining power (as well as the carbon dioxide or carbon monoxide content) of the blood to be made. The above described procedure has been found sufficiently accurate, however, to permit it to be used as a method of standardizing all of the simpler methods of determining the hemoglobin content of the blood for clinical purposes.

Quantitative Variations in Hemoglobin.—Haldane[16] found the mean oxygen combining capacity of the specimens of blood from the normal individuals he studied to be 18.5 volumes per cent. He called this 100 per cent. hemoglobin and this standard is now used clinically throughout the world. 18.5 volumes per cent., oxygen combining capacity, are the equivalent of 13.8 gms. of hemoglobin per 100 c.c. of blood. The following table shows the relation between grams of hemoglobin per 100 c.c. of blood, oxygen combining capacity in volumes per cent., and percentage hemoglobin as stated by Haldane.

Found	Factor	Result
Grams hemoglobin..........	1.34	Oxygen capacity
Grams hemoglobin..........	7.235	Per cent. hemoglobin
Oxygen capacity.............	5.4	Per cent. hemoglobin
Oxygen capacity.............	0.746	Grams hemoglobin
Per cent. hemoglobin........	0.185	Oxygen capacity
Per cent. hemoglobin........	0.138	Grams hemoglobin

Although Mayers and Butterfield, Williamson, Palmer and others have shown that the mean value for this hemoglobin content of the blood of normal adult males in this country is about 16.4 gms. per 100 c.c. of blood, it has seemed better to maintain the Haldane standard of 13.8 gms. of hemoglobin per 100 per cent. hemoglobin and to regard as normal values of 120 and 108 per cent. for adult males and females respectively.

Although the values for normal individuals are maintained fairly constant from about sixteen years of age to about sixty years of age, the normal values for hemoglobin in infants and children and in individuals past sixty are very different. These differences are shown in the diagram taken from Mayer's article[17] which includes Williamson's data[18] relative to hemoglobin content as well as data relative to erythrocyte counts at different ages. A scale showing percentage hemoglobin and the graph of the color index, mentioned below, have been added.
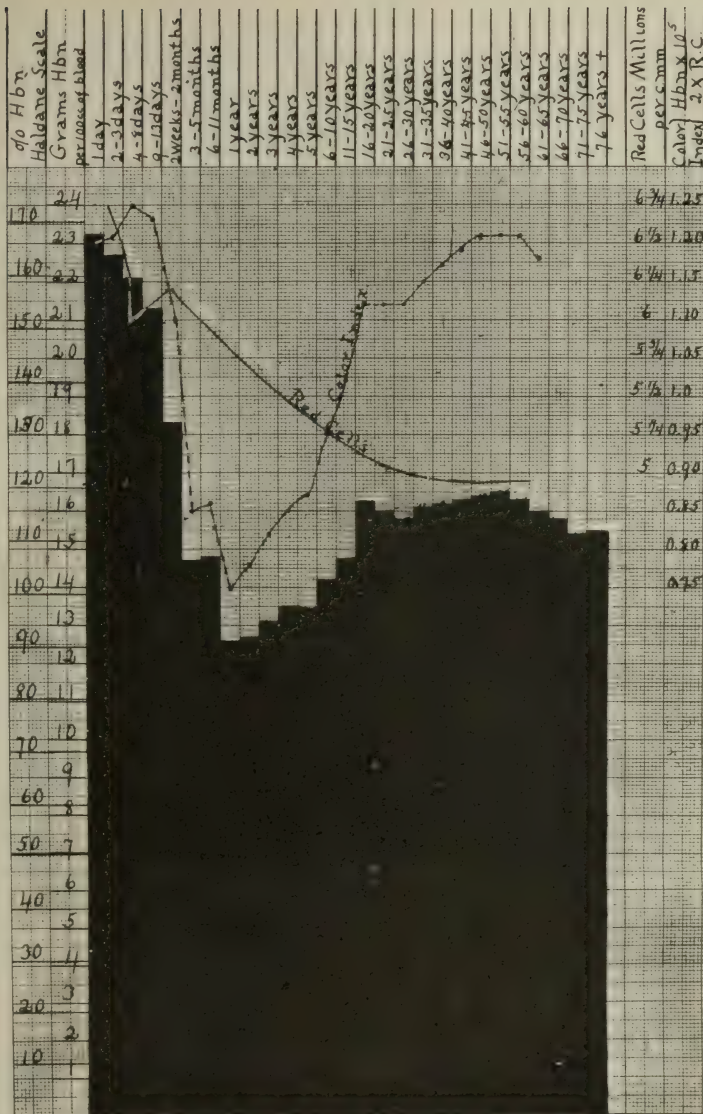
FIG. 145.—Chart showing age variations in the hemoglobin content of the blood, the erythrocyte count, the color index (a modification of Mayer's and Williamson's diagrams).

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Values for hemoglogin above 120 per cent. are rare after the first two months of life and are always associated with polycythemia. The principal conditions giving rise to supernormal hemoglobin values are thus those conditions giving rise to polycythemia mentioned above. (See discussion of red cell counts.) On the other hand, subnormal values for hemoglobin per cent. are common at all ages but unless the per cent. of hemoglobin drops below 80 a value slightly below the mean usually has no important significance except in the case of the newly born.

In other words, except in the case of the newly born, it is, as a rule, only hemoglobin values above 120 per cent. and below 80 per cent. that are of much clinical significance.

The Color Index.—Little is known about the pathological relation of hemoglobin to red cell count in infancy and childhood, but in adult life this relation is frequently one of the most important observations that can be made about the blood. Some idea as to whether the amount of hemoglobin in the red corpuscles is normal, increased or diminished can be gained by the study of the stained blood film but the quantitative expression of this is secured by calculating the "color index." This is done by multiplying the per cent. of hemoglobin by 10^5 and dividing this by twice the number of red corpuscles per cu. mm. of blood. The following table serves as an illustration.

Per cent. hemoglobin	Red cells per (mm.)^3	Color index
140	8,000,000	0.875
100	5,000,000	1.000
60	2,000,000	1.500

In the newly born and in adults the color index of normal individuals with values for hemoglobin contents and red cell counts that approximate the normal mean is about 1.2 and for infants and children it is about 0.9. As a rule, however, the color index is of importance, only in cases in which the percent-

age of hemoglobin is above 120 or below 80. The color index is usually less than 1.0 in conditions giving rise to a polycythemia, in anemia due to blood loss, in chlorosis and other conditions associated with anemia and insufficiency of iron in the food and in most types of so-called secondary or reversible anemia. It is usually normal in the anemias due to marrow aplasia. It is often above 1.0 in the leukemias and is practically always greater than 1.0 in the anemic phase of pernicious anemia, except that, in certain cases of pernicious anemia, the color index may be less than 1.0 in the early stages of remission. With this proviso in mind, from the point of view of the blood, the differentiations between pernicious anemia and other anemias often depends to a large extent on the color index.

CLINICAL STUDY OF THE COAGULATION MECHANISM

The Coagulation Time.—An accurate clinical method of determining the coagulation time of the blood is the one described by Lee and White,[19] a practical adaptation of Howell's method. The technique is as follows: Prepare three small test-tubes about 10 mm. in diameter by cleaning first with cleaning solution, then rinsing thoroughly with tap water and then with isotonic (0.85 per cent.) sodium chloride solution. Obtain blood from a vein in the usual manner allowing the blood to enter the syringe without suction and taking care to avoid air bubbles. Transfer at once to each tube about 1 c.c. of the blood. At the end of every minute gently incline the first tube until it is possible to invert the tube without any flow of blood occurring. Then incline the second tube every minute until the blood in that tube has coagulated. Then repeat the process with the third tube. The *longest* time interval between the first appearance of blood in the syringe and the definite coagulation of blood in one of the tubes is to be taken as the coagulation time. The coagulation time of the blood of normal adults when done by this method varies from about five to ten minutes.

Notes.—1. Air bubbles, foreign matter, agitation, all accelerate the process of coagulation and must be scrupulously avoided. A very slight inclination of the tube is all that is needed to determine whether or not the blood will flow.

2. Certain additional and sometimes important information can be obtained by the further observation of the coagulated blood. Stopper one of the tubes and place in the incubator for six hours or longer. Then note the following:

(1) Clot Retraction; the clot of normal blood should have retracted so that the clot forms a ball of fibrin suspended in more than an equal volume of clear serum.

(2) Observe whether any digestion of the clot has occurred as evinced by a moth-eaten appearance of the clot, fibrinolysis of Goodpasture, seen especially in the blood of persons suffering from cirrhosis of the liver.

(3) Note the color of the serum. The serum of normal individuals will be of a pale straw color. After a meal it may be somewhat opaque owing to the presence of suspended fat. Any increase in the depth of the color is called pleiochromia. The degree of pleiochromia may be determined quantitatively by diluting the serum with distilled water until the serum is colorless. In the normal person it ordinarily requires not more than thirty dilutions with distilled water to render the serum colorless. In cases of pleiochromia several hundered dilutions may be required. For clinical purposes it is usually not necessary to determine quantitatively the extent of the pleiochromia; it is sufficient to note whether the pleiochromia is slight, moderate or marked. Serum pleiochromia due to the presence of excessive quantities of bile pigments is seen in cases of obstructive jaundice and in cases of increased intravascular hemolysis—streptococcus hemolyticus septicemia and perhaps in sepsis due to other causes if severe, in polycythemia vera, hemolytic jaundice, malaria, pernicious anemia, etc. But serum pleiochromia may of course be due to other pigments and occur in other conditions, *e.g.* carotinemia. (For methods of determining the bile pigments in blood see especially the article by Blankenhorn.[20])

3. It is sometimes impossible to obtain blood from a vein. In such cases capillary blood obtained from puncture of the skin in the usual manner must be used to determine the coagulation time. One may make use of the apparatus described by Brodie and Russell.[21] The apparatus consists essentially of a truncated glass cone which fits into a chamber provided with an inlet tube. The test is carried out as follows: A drop of blood obtained with the usual precautions is immediately secured on the tip of the cone and the latter is inverted in the chamber which is then placed on the stage of a microscope. The process of coagulation is observed through a low power objective lens. The end point is determined as follows: At the end of every thirty seconds air is forced

into the chamber by means of a small rubber bulb attached to the inlet tube. As long as the blood remains uncoagulated the inrush of air will cause agitation of the corpuscles in the drop. When this no longer occurs coagulation is regarded as having taken place. A coagulation time of eight minutes or over, when determined by this method, is pathological.

4. Rodda[22] has devised an even simpler method of determining the coagulation time. The apparatus consists of two watch glasses $1\frac{1}{2}$ inches in diameter and a No. 6 lead shot. The skin is punctured to a depth of about 0.5 cm. by a scalpel to secure an even flow of blood. The first drops of blood are wiped away and a drop is then received in one of the watch glasses containing the lead shot. The second watch glass is inverted over this and the two are gently tilted every thirty seconds until the shot no longer rolls about but is held by the clot. It is essential, of course, that the watch glasses and shot be clean and that any undue agitation be avoided. The coagulation time when determined by this method varies from five to ten minutes in the case of normal adults.

5. The coagulation time as determined by any of the above methods is frequently slightly prolonged, twelve to fifteen minutes, during the first few days of life and this prolonged coagulation time may be associated with pathological hemorrhages.

6. Addition of tissue juice to the blood hastens the process of coagulation. If, therefore, the coagulation time is actually only slightly prolonged it is possible that it may be found to be within normal limits when determined by a method that makes use of blood contaminated by tissue juice. (For a consideration of theories of coagulation the reader is referred to the articles by Nolf,[23] Howell[24] and Lee and Vincent.[25])

7. Another coagulation test useful in studying patients showing jaundice due to obstruction is the "calcium in vitro test" introduced by Lee and Vincent.[26] This test is carried out by adding three drops of a 1 per cent. solution of calcium chloride to one of the small test tubes mentioned above before adding 1 c.c. of blood. The addition of this amount of calcium to normal blood and to blood showing a delayed coagulation time due to causes other than obstructive jaundice does not influence the coagulation time. In the case of individuals with obstructive jaundice, however, in whom the coagulation time is prolonged then the addition of this amount of calcium usually brings the coagulation time within normal limits. The test may be regarded as a test for a diminution in the amount of available calcium in the blood and if positive constitutes an indication for the administration of calcium to the patient.

The Bleeding Time.—This simple test described by Duke[27] may yield very important information in certain cases. It is performed as follows. Puncture the lobe of an ear with a sharp

needle so that a definite flow of blood is obtained. With a
piece of filter paper absorb the blood after every fifteen seconds.
When bleeding has ceased count the number of drops on the
filter paper and thus calculate the bleeding time. The bleed-
ing time of normal adults varies from one to three and a half
minutes. The bleeding time of normal infants may be as long
as five minutes during the first week of life.

**The Interpretation of the Platelet Count, Bleeding
Time and Coagulation Time.**—The coagulation of the blood
is normally dependent upon the agglutination of the platelets.
Presumably, under ordinary circumstances, in normal indivi-
duals, platelets are constantly being agglutinated in small
numbers yielding so-called thromboplastic substance to the
tissues where it seems to be stored up to a certain extent.
Moreover, when blood is shed the amount of fibrin which is
formed in a given time is proportional to the number of
platelets that have agglutinated. The number of platelets
in the circulating blood of a normal individual varies from
200,000 to 400,000 per cu. mm. Any diminution below
200,000 per cu. mm. is called thrombopenia and any increase
over 400,000 is called thrombocytosis. In any condition
in which there is a thrombopenia but in which the quality
of the platelets is not changed, *e.g.* idiopathic purpura
hemorrhagica (essential thrombopenia), aplastic anemia,
lymphatic leukemia, pernicious anemia, etc., certain character-
istic signs appear depending on the number of platelets present
in the circulating blood. If the count is above 100,000 per cu.
mm. there will probably be no symptoms. The bleeding time
will probably be within normal limits. The coagulation time
will be normal and the clot retraction fairly good.

If the number of platelets is somewhat lower than 100,000
per cu. mm., say 60,000 to 80,000, there may still be no symp-
toms but the bleeding time will probably be slightly prolonged.
The coagulation time will be normal but the clot retraction will
be poor.

If the number of platelets is lower than 60,000 per cu. mm.,
say 30,000 to 50,000, ecchymoses and petechiae will almost

surely be found and perhaps there will be spontaneous hemor-
rhages from mucous membranes. The bleeding time will be
somewhat prolonged, perhaps to a half-hour or more. The
coagulation time may be normal but the clot retraction and
quality of the clot will be very poor.

If the number of platelets is below 30,000 per cu. mm. there
will be not only ecchymoses and petechiae but almost surely
hemorrhages from mucous membranes. The bleeding time
will be prolonged indefinitely. The coagulation time may be
very slightly but not markedly prolonged. There will be no clot
retraction.

The explanation as to why it is that in cases showing a
thrombopenia the bleeding time is prolonged but the coagula-
tion time is not prolonged is probably as follows: The number of
platelets being small the tissues are poorly endowed with throm-
boplastic substance, and hence when an ear is pricked the blood
continues to ooze. When blood is taken from a vein, although
the number of platelets is small, nevertheless they are active
and are present in sufficient numbers to form a few strands
of fibrin in the ordinary course of time. This small amount of
fibrin, is, however, sufficient to prevent the escape of blood
when the tube is inverted.

Sometimes there is an increase in the number of platelets
in the circulating blood, thrombocytosis, without at the time
any alteration in their quality as after severe hemorrhage,
after splenectomy, and in so-called idiopathic thrombosis.
In such cases thrombi may be formed but there may be no
change in the bleeding time or coagulation time and the quality
and retraction of the clot may be normal.

Sometimes the number of platelets in the circulating blood is
normal or above normal but the quality of the platelets is
apparently altered—hemophilia. In such cases it appears
that the platelets agglutinate or yield up their thromboplastic
substance very slowly. As a result of this the coagulation
time is greatly prolonged but the clot, when it does form, is
normal and retracts well. On the other hand, such cases
usually show a normal bleeding time inasmuch as the tissues

are endowed with the normal amount of thromboplastic substances.

The coagulation and bleeding times may be prolonged in conditions in which there is no clearly demonstrable qualitative or quantitative alteration in the platelets. The coagulation time or bleeding time is especially apt to be prolonged in any case of severe and prolonged jaundice, in Henoch's purpura, in hemorrhagic disease of the newly born, in anaphylactic shock, in polycythemia vera and in the leukemias. In these conditions pathological hemorrhages may occur. Slight variations from the normal have been noted also in certain acute infectious fevers, notably lobar pneumonia, influenza and typhoid fever, and in certain cases of chronic infection of the head sinuses and tonsils. The mechanisms by which these disturbances are effected are not known, except that, as mentioned above, there seems to be a lack of *available* calcium in the blood of certain persons showing jaundice due to obstruction. (See the article by Lee and Minot.[28])

"SPECIAL METHODS OF EXAMINATION OF THE BLOOD"

The Percentage Volume of the Red Corpuscles.—It is frequently desirable to know what per cent. of the total volume of blood is made up by the red blood corpuscles. This figure is known as the percentage volume of red corpuscles and may be determined by means of a hematocrit prepared as follows: Two thick-walled graduated tubes about 5 mm. in diameter and 8 cm. long are selected and sealed at one end. They are then calibrated by letting in water, 0.02 c.c. at a time, from a previously calibrated microburette.

The blood to be examined is obtained and placed in the calibrated tubes as follows: Approximately 0.05 gm. of powdered potassium oxalate are placed in a test tube and covered with a layer of liquid albolene about 1 cm. thick. A similar layer of liquid albolene is placed in each of the two calibrated tubes and also in a 10 c.c. glass syringe. A long sterile No. 20 needle

is then attached to the syringe and inserted into a vein in the usual manner and about 9 c.c. of blood removed, care being taken not to admit air to the syringe. After withdrawal from the vein the tip of the needle is placed beneath the layer of albolene in the test tube and the blood forced into the test tube under the albolene. The blood is then thoroughly stirred with a glass rod in order to bring it in contact with the anticoagulent, care being taken to introduce no air to the blood. Enough blood to fill both of the calibrated tubes is drawn up into a syringe containing albolene and transferred to the calibrated tubes. These tubes are then mounted in cork stoppers, placed in the ordinary brass containing tubes and centrifuged for one half hour at about 2000 revolutions per minute. The proportion of red corpuscles to the whole blood is then read off from each tube and the average taken. The percentage volume of red cells when determined in this manner ranges from about 38 to 42 c.c. of cells per 100 c.c. of blood in the case of normal adults.

Notes.—1. Admirable hematocrit tubes can be made from a microburette such as the one described by Folin and McEllroy[29] in their method of determining sugar in the blood.

2. Aeration of the blood permits the escape of carbon dioxide and the subsequent passage of chloride ion from corpuscles to plasma. This causes a slight shrinkage of the corpuscles. Unless great accuracy is desired or unless one is making a determination of the blood gases or of the quantity of the chloride ion in the blood plasma the use of liquid albolene may be dispensed with and 0.05 c.c. (one drop) of a 60 per cent. solution of sodium citrate may be added to the blood in place of the powdered potassium oxalate.

3. The percentage volume of the red corpuscles may be estimated conveniently, for clinical purposes, by the method of Larrabee.[30] In this method blood obtained by puncture of the skin with a lancet is allowed to flow into a small mixing tube (20 × 8 mm.) containing about 3 mgm. of powdered sodium oxalate. A volume of $\frac{1}{3}$ c.c. is marked off on the tube and the blood is allowed to flow up to this mark. After mixing with sodium oxalate the blood is at once aspirated by means of an ordinary dropper bulb into a sedimentation tube made of thick-walled barometer tubing about 10 cm. long and about 2 mm. in diameter and slightly drawn out at one end. When the sedimentation tube has been nearly filled with

blood the tip is removed from the mixing tube and covered with a finger. The dropper bulb is now removed and the tube is inverted to allow the blood to flow to the other end of the tube. Both ends of the tube are sealed by means of an elastic band and the tube is kept in a vertical position until complete sedimentation has occurred. This will require from one to three days. When it is evident that no further sedimentation will occur readings are made. By means of a millimeter rule the height of the entire column of fluid and the height of the column of corpuscles are measured and the percentage volume of corpuscles calculated in the usual manner. The percentage volume of corpuscles when determined by this method is about 50 in normal individuals. The volume index is calculated by multiplying the percentage volume of cells by 10^5 and dividing the product by the number of red corpuscles per cu. mm. of blood.

The Volume Index.—It is often useful to know the mean relative volume of the red corpuscles and for this purpose one has recourse to the calculation of the "volume index," described by Capps.[31] The volume index of the red corpuscles is calculated by multiplying the percentage volume of red corpuscles by 1.25×10^5 and dividing this product by the number of red corpuscles per cu. mm. of blood. Thus, for example, if the percentage volume of red corpuscles were 20 and the number of red corpuscles were 2,500,000 per cu. mm. the volume index would be $\dfrac{20 \times 1.25 \times 10^5}{2,500,000} = 1.0$.

The volume index of normal adults varies but slightly from 1.0.

Very little is known about the significance of variations from the normal mean volume of the red corpuscles in infants and children. In adults, large red cells are seen frequently in pernicious anemia, sometimes in the leukemias and in erythremia and rarely in other conditions. Red cells of normal size are the rule in anemia due to aplasia and smaller than normal red cells are often a feature of anemia due to blood loss and hemolytic anemias other than pernicious anemia.

The Volume of the Blood.—A very simple method for determining the volume of the blood is the one making use of the dye, vital red, introduced by Keith, Rowntree and Geraghty.[32] The method is as follows:

1.5 gms. of vital red are dissolved in 100 c.c. of distilled water and 20 c.c. of this solution are placed in each of five 100 c.c. flasks. The flasks are stoppered with cotton, covered with tinfoil and heated in the autoclave at a pressure of 5 lb. for thirty minutes. The amount of solution in each flask suffices for one determination of blood volume. Similar quantities of 0.5 per cent. sodium chloride solution are prepared and sterilized. An amount of the solution of the dye, depending on the weight of the patient (0.2 c.c. per Kg.), is drawn up into a sterile syringe previously rinsed with salt solution.

About 10 c.c. of blood are then withdrawn from a vein into a syringe in the usual manner. The syringe is disconnected from the needle, which remains in the vein, and handed to an assistant who transfers the blood to a centrifuge tube containing 0.05 c.c. of 60 per cent. sodium citrate solution.

The syringe containing the dye is now attached to the needle in the vein and the contents of the syringe are slowly expressed. The last traces of dye are washed into the vein by drawing up blood into the syringe and then expressing the blood into the vein again.

At the end of three minutes 10 c.c. of blood are again withdrawn and transferred to a centrifuge tube containing 0.05 c.c. of 60 per cent. sodium citrate solution. The blood specimens in the centrifuge tubes are thoroughly stirred and a specimen from each placed in a small hematocrit tube. The two hematocrit tubes and the two centrifuge tubes are then centrifuged at about 2000 revolutions per minute for thirty minutes. The percentage volume of cells in each of the hematocrit tubes is read off and the average taken. The supernatant plasma from each of the centrifuge tubes is removed and treated as follows:

Exactly 0.5 c.c. of the 1.5 per cent. dye solution are diluted to 100 c.c. with 0.5 per cent. sodium chloride solution. To 2 c.c. of plasma obtained before injection of the dye are then added 2 c.c. of the diluted dye solution and 4 c.c. of 0.5 per cent. solution of sodium chloride. The fluids are mixed, and placed in the left hand cup of a colorimeter which is set at the 20.0 mark. This is the standard.

To 2 c.c. of the plasma obtained after injection of the dye, 6 c.c. of 0.85 per cent. sodium chloride solution are added and the fluids mixed. This is the test solution, and is entirely comparable with the standard. It is placed in the right hand cup of the colorimeter and compared with the standard. If the standard is set at 20.0 then the following equation holds:

$$C \times U \times 10 = \text{c.c. of plasma.}$$
Where C = No. c.c. of dye solution injected.
and U = reading of test solution.

Notes.—1. The site of the injection may be anaesthetized by the intradermal administration of a few minims of a 1 per cent. novocain solution.

2. In the case of infants and young children it is desirable to dilute the dye to be injected with salt solution. The required amount of dye, 0.2 c.c. per kilogram of body weight, is placed in a sterile evaporating dish and drawn up into the syringe. 2 c.c. of sterile 0.8 per cent. sodium chloride are then placed in the dish and drawn up into the syringe. This procedure is repeated until the volume of diluted dye in the syringe is about 12–14 c.c. The reason for so diluting is that a loss of, say, one drop from 2 c.c. of solution would cause an error of about 2.5 per cent. but the loss of one drop from 10 c.c. would cause an error of only about 0.5 per cent. Even when the volume of dye to be injected is 10 or 12 c.c. it is worth while to place the dye in a dish and wash it into the syringe by drawing up through the needle several 2 c.c. portions of salt solution.

3. Some operators prefer to allow salt solution to run through the needle into the vein slowly and continuously during the three minute interval between the time that the dye is injected and the blood for examination is withdrawn. The purpose of this procedure is to prevent coagulation in the needle. Its disadvantage is that the needle may become dislodged during the process of connecting or disconnecting the syringe.

4. The dye is eliminated from the body so slowly that not more than one determination of blood volume should be made in one day.

5. Following a determination of the blood volume by this method a very mild febrile reaction lasting a few hours may rarely occur. The cause of this reaction is unknown. It may be avoided by keeping the patient in bed for a period of from six to eight hours following the injection of the dye.

6. The site of the injection must be inspected the day following the determination. If a pink areola is observed the results must be discarded inasmuch as it cannot be determined whether this areola is caused by leakage of the dye during the injection or leakage from the vein after the needle has been withdrawn. The pink areola is of no consequence to the

patient's well-being and disappears completely in the course of 8–10 days.

7. Vital red, especially prepared for the determination of blood volume may be obtained from the National Aniline & Chemical Company, 40 Rector Street, New York, N. Y.

As determined by this method the volume of the plasma is maintained with remarkable constancy at about 50 c.c. per kg. of body weight in the case of normal resting adults and in many pathological conditions (Bock). It is the same in older children. In infancy the plasma volume is somewhat higher than this and by no means so nearly constant (Lucas). The plasma volume is reduced immediately after the loss of much blood and may be somewhat reduced in lobar pneumonia and other febrile conditions, and in cases of dehydration in infants. It is often increased in pernicious anemia (Denny) and perhaps in other conditions with which a chronic severe anemia is associated. It may be increased in certain cases of erythremia.

It would appear that the plasma volume in a given individual is a much more nearly constant factor than the cell volume and hence more nearly constant than the whole blood volume. The latter, in normal resting adults, is about 83 c.c. per kg. of body weight or about one-twelfth or one-thirteenth of the weight of the body.

Resistance of the Red Corpuscles.—Various reagents have been used to test the osmotic resistance of the red corpuscles: sodium chloride, sodium oleate, saponin, hemolytic serum —but the one most commonly employed in clinical laboratories is hypotonic aqueous sodium chloride solution. The determination is carried out as follows:

Into each of a series of 20 test tubes measuring about 10 × 75 mm. are placed 2 c.c. of hypotonic sodium chloride solution ranging in concentration from 0.60 per cent. to 0.22 per cent. at intervals of 0.02 per cent.

Sufficient blood to yield about 6 c.c. of red corpuscles obtained from a vein in the usual way is transferred to a flask containing an equal volume of 0.85 per cent. sodium chloride solution to which has been added 0.05 c.c. of 60 per cent. sodium citrate solution for each 10 c.c. of blood withdrawn. The blood is

then well mixed with the diluting fluid and centrifuged, and the supernatant fluid removed.

Two-tenths of 1 c.c. of red cells are then added to each tube of the series of tubes containing hypotonic sodium chloride solution and the cells and solution are mixed by inverting the test tubes several times.

A control series of 10 tubes is now prepared as follows: 1 c.c. of red corpuscles is added to 10 c.c. of distilled water
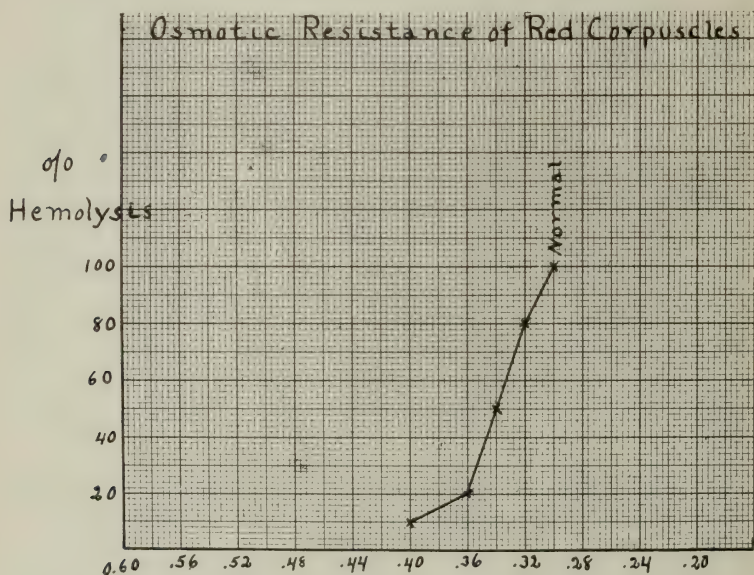
FIG. 146.—Concentration NaCl.

in a test tube and the cells laked by thorough shaking. Into the first of the control series of tubes are placed 0.2 c.c. of the solution of laked red corpuscles and 1.8 c.c. of distilled water. This tube represents a depth of color corresponding to the depth of color that would be produced if 10 per cent. of the red cells in any of the tubes of the test series were hemolyzed. Into the second tube are put 0.4 c.c. of the solution of laked red corpuscles and 1.6 c.c. of distilled water. This gives a color corresponding to hemolysis of 20 per cent. of cells. To the remaining tubes are added enough of the solution of laked red

corpuscles and distilled water to give depths of color corresponding to hemolysis of 30, 40 ,50, 60, 70, 80, 90 and 100 per cent. of the red cells. All tubes are now placed in the ice-chest. At the end of $\frac{1}{2}$ hour they are again shaken and returned to the ice-chest for one hour. Readings should then be made.

The maximum concentrations of sodium chloride in which any hemolysis occurs and in which hemolysis is complete are noted as well as the percentage of cells hemolyzed in each tube lying between the tube in which the least hemolysis is observed and the tube containing the highest concentration of salt solutions in which hemolysis is complete. The results may be recorded conveniently by plotting a graph on coördinate paper. The graph for normal blood is shown in the accompanying diagram.

Notes.—1. It is of the utmost importance that the glassware with which the blood comes in contact should be chemically clean, otherwise one cannot be sure that hemolysis is not caused by influences other than osmosis. A relatively slight increase in the acidity of the salt solution may cause hemolysis even in high concentrations of sodium chloride. On this account, the small test tubes after treating with cleaning solution should be rinsed many times with tap water and finally with distilled water and then allowed to dry before being used.

2. Solutions of sodium chloride if placed in containers made of "non sol" glass and thoroughly shaken each time before use should keep indefinitely. Even so, it is desirable that a control resistance test with normal red corpuscles should be made whenever red cells of unknown resistance are to be tested.

3. In case it is impossible to obtain blood from a vein a rough idea of the resistance of the red corpuscles may be obtained by mixing directly with hypotonic salt solutions drops of blood obtained by puncture of the skin.

In this case a small quantity of salt solution, 1 c.c. or 0.5 c.c. should be used. A control test with normal red corpuscles should be made in every instance.

4. Instead of determining the percentage of cells hemolyzed by color comparison a sufficiently accurate idea of the extent of hemolysis for most clinical purposes may be obtained by noting the residue of cells in the various test tubes in which hemolysis has occurred.

In the case of normal adults hemolysis almost never occurs in a concentration of sodium chloride greater than 0.42 per cent.

and is almost always complete in a concentration of sodium chloride not less than 0.28 per cent. The hemolytic span or difference in concentration between the greatest concentration in which any hemolysis occurs and the greatest concentration in which complete hemolysis occurs almost never exceeds 0.12 per cent. Moreover, 50 per cent. of the cells are nearly always hemolyzed at a concentration of sodium chloride not more than 0.06 per cent. less than the highest concentration in which the smallest amount of hemolysis occurs.

In the case of pathological red corpuscles the fragility graph may be displaced outside of normal limits. An increased fragility or diminished resistance, shift to the left (Minot), is represented by a displacement of the graph to regions of higher concentrations of sodium chloride. Diminished fragility or increased resistance, shift to the right, is represented by a displacement of the graph to regions of lower concentrations of sodium chloride. Moreover, a flattening of the graph may occur at either end, "trickling effect," or a diminished or increased hemolytic span may be noted.

Presumably red corpuscles which are hemolyzed in high concentrations of sodium chloride solution are more readily destroyed in the body than those which are hemolyzed in low concentrations of sodium chloride. It has been suggested, moreover, that in the absence of influences abnormal in degree or kind tending to diminish the natural biological resistance of red corpuscles (e.g. the hemocatatonistic influence of spleen in hemolytic jaundice) the older red corpuscles are more fragile than the younger ones.

Although proofs of these conceptions of the physiological significance of altered resistance of the red corpuscles are wanting, they serve as a basis for interpreting and correlating the results of fragility tests. Thus in conditions in which one might expect to find many cells of different ages, polycythemia, a lengthening of the hemolytic span with perhaps a "trickling effect" starting at high concentrations of sodium chloride is often observed. In conditions giving rise to marrow aplasia resulting ultimately in the presence of cells in the peripheral blood of

approximately the same age a narrowing of the hemolytic span is observed, a finding that suggests an unfavorable prognosis.

In acutely developing anemia due to blood loss one would expect a relative increase in immature red cells in the blood due to marrow stimulation and one often finds in such conditions a lengthening of the hemolytic span with a shift of the graph to the right (Minot).

A shift of the graph to the left (increased fragility) and a lengthened hemolytic span with or without a "trickling effect" constitute almost a sine qua non of hemolytic jaundice and are seen also in those cases of pernicious anemia that simulate hemolytic jaundice (Minot).

It is often said that the red corpuscles from cases of pernicious anemia show an increased resistance to hypotonic sodium chloride solution. This finding is by no means a constant one and when it is observed the increased resistance is but slight.

Increased resistance of the red corpuscles is a feature of the blood of dogs following splenectomy (Krumbhaar) and the same finding has been observed in man following splenectomy. But the resistance of the red corpuscles usually returns very slowly to normal following splenectomy in cases of hemolytic jaundice.

Hemocidal Properties of Blood Serum.—The determination of the osmotic resistance of the red corpuscles to hypotonic sodium chloride solution affords no information, of course, concerning the alterations in the blood serum which may, in vivo, predispose to abnormal red cell destruction. Different blood sera may be compared in this respect by means of the test introduced by Buckman and Horrall.[33] This is performed as follows:

Enough blood to yield at least 16 c.c. of serum and 2 c.c. of red corpuscles is collected in a chemically clean, dry cylinder and defibrinated by stirring with a glass rod. The mixture is then filtered and centrifuged and the supernatant serum removed.

A series of dilutions of serum with distilled water in small test tubes is then made up as follows: Into the first tube are

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placed 0.95 c.c. of serum and 0.05 c.c. of distilled water; into the second tube 0.9 c.c. of serum and 0.10 c.c. of distilled water; into the third tube 0.85 c.c. of serum and 0.15 c.c. of distilled water, and so on, the last tube containing 0.05 c.c. of serum and 0.95 c.c. of distilled water.

To each tube is then added 0.1 c.c. of red cells and the tubes shaken, stoppered and placed in the ice-chest for four hours. At the end of this time readings are made, the per cent. of hemolysis in each tube being gauged by means of a series of color standards prepared as directed in the description of the determination of the resistance of red cells to hypotonic sodium chloride solution.

Ten cubic centimeters of a mixture of serum and distilled water containing the same relative volumes of serum and dis-tilled water present in the tube which contained the greatest amount of serum of any tube in which hemolysis occurred are now made up. The depression of the freezing point of this mixture is determined in the usual manner by means of a Beckmann thermometer. The freezing point depression of a mixture corresponding to the one in which complete hemolysis occurred in the presence of the greatest amount of serum is calculated. These results are then expressed in terms of per-centage concentration of sodium chloride in order to render them comparable with results obtained in the ordinary resistance test.

Notes.—1. If sufficient blood is available similar tests may be made with a mixture of the patient's serum and the red cells of a normal person of the same isohemolytic group and also with a mixture of the red cells of the patient and the serum of a normal individual of the same isohemolytic group.

2. It often happens that the amount of blood obtainable is insufficient to carry out the entire test. However, the maximal concentration of soluble serum constituents in which any hemolysis occurs may be determined with the use of only 5 c.c. of serum and in case a thermocouple is available the quantity of serum needed for determining the freezing point depression of the mixture of serum and distilled water will not be over 1 c.c.

3. The Beckmann thermometer should be read to 0.001° C. although it is probable that unless special corrections for volume hysteresis of the

glass, heat of stirring, temperature of the room, etc., are made, the freezing point depression cannot be determined with an accuracy greater than 0.005° C.

4. Ordinary distilled water is not sufficiently pure to be used in this test. Tap water should be distilled three times using a tin condenser.

5. Calibrated microburettes such as are used in the Folin and McEllroy[29] method of determining sugar in the blood serve admirably for the purposes of this test.

6. Calculations. In solutions of electrolytes of concentrations corresponding to the range within which the serum dilutions will usually fall it may be assumed; (1) that the depression of the freezing point of the mixture is directly proportional to the quantity of serum present and (2) that a freezing point depression of 0.010° C. corresponds to a 0.0168 per cent. solution of sodium chloride. (Strictly speaking this proportionality does not hold exactly because as the solution becomes more dilute the electrolytes become more dissociated and the actual value of the freezing point depression becomes greater than the calculated value.)

Example of calculation. Suppose the least hemolysis was observed in a mixture of 0.45 c.c. of serum and 0.55 c.c. of distilled water and suppose the freezing point depression of such a mixture was 0.319° C. Suppose also that complete hemolysis was first observed in a mixture of 0.30 c.c. of serum and 0.70 c.c. of distilled water. Then

Maximal equivalent concentration of sodium chloride in which any hemolysis occurred = 3.19 × 0.0168 = 0.54 per cent.

Probable freezing point depression of second mixture = 0.3 × 0.319 ÷ 0.45 = 0.213°.

Maximal equivalent concentration in sodium chloride in which complete hemolysis occurred = 2.13 × 0.0168 = 0.36 per cent.

Hemolytic span = 0.54 − 0.36 = 0.18 per cent. NaCl.

In normal individuals the limits for maximal equivalent concentration of sodium chloride in which any hemolysis may occur are 0.50 per cent. and 0.42 per cent. and the limits for maximal equivalent concentration of sodium chloride in which complete hemolysis may occur are 0.32 per cent. and 0.20 per cent. The maximal normal hemolytic span corresponds to 0.20 per cent. NaCl and the minimal normal hemolytic span corresponds to 0.16 per cent. NaCl.

The occurrence of hemolysis in higher concentrations than those set by the normal limits is the rule in hemolytic jaundice, streptococcus hemolyticus septicemia, and pernicious anemia

in relapse and is seen often in erythremia. In the latter condition an abnormally long hemolytic span is a frequent occurrence (Minot and Buckman).

Hemolysis occurs within normal limits and the hemolytic span is normal in cases showing a chronic anemia of the "secondary" type.

In severe acutely developing anemia due to blood loss hemolysis occurs in lower than normal concentrations and the hemolytic span may be shortened.

Determination of Isoagglutination Group.—In the case of human blood four isoagglutination groups are distinguished, I, II, III and IV. In the classification described by Jansky[34] Group I is that group in which the serum agglutinates the red cells of all other groups and in which the red cells are agglutinated by the serum of no group. Group IV is the group in which the serum agglutinates no red cells and in which the red cells are agglutinated by the serum of all the other groups. Groups II and III are those groups in which the serum agglutinates the red cells of Group IV and either the cells of Group III or II respectively and in which the red cells are agglutinated by the serum of Group I and by the serum of either Group III or Group II respectively. The agglutination reactions of the serum and red cells of the different groups are shown in the following diagram.

Serum of group	Red cells of group			
	I	II	III	IV
I	o	+	+	+
II	o	o	+	+
III	o	+	o	+
IV	o	o	o	o

o = No agglutination.
+ = Agglutination.

Landsteiner has suggested that the existence of these groups can be explained by assuming that there exist two agglutinogens, A and B contained in the red cells and two corresponding agglutinins a and b contained in the serum. In the Jansky system the red cells of group IV contain agglutinogens A and B but the serum contains no agglutinins. The red cells of Group II contain agglutinogen A and the serum of Group II contains agglutinin b. The red cells of Group III contain agglutinogen B and the serum of Group III contains agglutinin a. The red cells of Group I contain no agglutinogens but the serum of Group I contains both agglutinins, a and b.

It has been shown also that human blood may be divided into four isohemolytic groups, strictly parallel to the four isoagglutination groups. Although red cells which contain either agglutinogen or both agglutinogens always contain the corresponding hemolysinogen or both hemolysinogens, in the majority of cases the serum is wanting in hemolysin. In other words, hemolysis never occurs in the absence of agglutination but agglutination may, and in fact usually does, occur without hemolysis.

Technique of Determining the Isoagglutination and Isohemolytic Group of an Individual.—An amount of blood sufficient to yield 2 c.c. of red corpuscles is drawn into twice its volume of 0.85 per cent. sodium chloride solution to which has been added 0.05 c.c. of 60 per cent. sodium citrate solution for each 10 c.c. of blood drawn. The blood is mixed with this solution, centrifuged and the supernatant fluid withdrawn. The cells are then washed once with four times their volume of 0.85 per cent. sodium chloride solution and a 5 per cent. suspension of these washed cells is made up.

In each of three clean test tubes (about 10 × 75 mm.) are then placed 0.5 c.c. of the 5 per cent. suspension of red corpuscles. To the first of these tubes are added 0.5 c.c. of Group II serum, to the second 0.5 c.c. of Group III serum and to the third 0.5 c.c. of 0.85 per cent. sodium chloride solution (control). The contents of the tubes are mixed, the tubes stoppered and allowed to stand at room temperature for one hour. At the end

of this time observations are made. One notes directly whether any hemolysis has occurred and by examining the cells under a microscope whether any agglutination has occurred.

From a glance at the diagram it is obvious that the individual is a member of isoagglutination Group I if no agglutination has occurred, of Group III if agglutination has occurred only in the case of the mixture containing Group II serum, of Group II if agglutination has occurred only in the case of the mixture containing Group III serum, and of Group IV if agglutination has occurred in both cases.

Notes.—1. It is frequently necessary in investigative work to substantiate the results obtained through the above described technique by testing the cells of the individual in question against the sera of Groups I and IV and by testing the serum of the individual against the cells of all the groups.

2. Preparation of test sera. These are easily prepared by withdrawing convenient amounts of blood (50 c.c. or more) from individuals known to be of Group II and Group III and allowing the blood specimens to clot. The sera are then removed by means of a pipette, and transferred to small ampules holding 0.5 c.c. The entire procedure should be carried out under conditions of strict asepsis. Even so it is desirable to add tricresol to the extent of 0.05 c.c. to each 10 c.c. of serum. The test sera may keep indefinitely but it is desirable to test each lot for potency at the end of every two months.

3. In clinical practice it is customary to employ a simplified technique for blood grouping. Several procedures have been described. The microscopic method of Minot[35] is carried out essentially as follows: Place a drop of 1 per cent. sodium citrate on a clean slide and to this add a drop of the blood from the individual whose group is to be determined. Mix. Mark with a glass pencil one end of a clean glass slide with the figure 2, and the other end with figure 3. Place a drop of Group II serum on the end marked 2, and a drop of Group III serum on the end marked 3. By means of an applicator mix one drop of the citrated blood with the drop of Group II serum. By means of another applicator mix another drop of the citrated blood with the drop of Group III serum. Cover with cover-slips and examine with high dry objective lens. Cover with a piece of moist filter paper and examine again after one hour. Before examining the second time tap the cover-slips with an applicator in order to break up any artificial clumps or rouleaux. If neither serum causes any agglutination the blood examined is in Group I. If Type III serum causes agglutination but Type II serum does not, the blood examined is in

Group II. If Type II serum causes agglutination but Type III serum does not the blood examined is in Group III. If both sera cause agglutination the blood examined is in Group IV. Instead of mixing the blood with sodium citrate solution sodium citrate to the extent of 1.5 per cent. may be added to the test sera and the blood mixed directly therewith (Vincent). Agglutination may be observed macroscopically in most cases, but in any doubtful case resort to microscopic examination must be made.

4. Slightly more than one half of all individuals are members of Group I and somewhat less than half members of Group II. Individuals belonging to Groups III and IV are rare, members of Group IV being the rarest of all.

5. The Jansky classification of groups has been described because it has been recommended for universal adoption by special committees representing the American Association of Immunologists, the Society of American Bacteriologists and the Association of Pathologists and Bacteriologists.[36] It should be emphasized, nevertheless, that Moss's[37] classification is much more generally in use than Jansky's throughout the Americas and Western Europe. The Moss System and the Jansky System are identical, however, except that Groups I and IV are interchanged.

6. In terms of the technique above described it turns out that the group of an individual is sometimes determined at birth, usually at the age of one year, and always at the age of two years. The agglutinogens are almost if not always present at birth (Descatella and Sturli) and B. B. Jones[38] has shown that with the use of a special technique permitting the detection of hemagglutinins and hemolysins in low concentration it is possible to detect the presence of isoagglutinins in the great majority and isohemolysins in over one-fourth of newly born infants. It would seem that the reported rarity of reactions following transfusion of newly born infants with blood where compatibility has not been determined may be due to the low concentration of isohemolysins in the infantile blood. But the possible presence of isochemolysins in concentration sufficient to hemolyze the infused red corpuscles in any given newly born infant makes it necessary to determine the compatibility of the donor's blood and the infant's blood if one is to preclude with certainty the danger of a hemolytic reaction.

7. It is not necessary that the group of donor and recipient be known in order to determine blood compatibility. The so-called direct test may be applied. This is carried out as follows: 0.5 c.c. of a 5 per cent. suspension of the washed red cells of the recipient are mixed with 0.5 c.c. of 0.85 per cent. sodium chloride solution (control) and another 0.5 c.c. of the 5 per cent. suspension of washed red cells of the recipient are mixed with 0.5 c.c. of the serum of the donor. In a similar manner, 0.5 c.c. of a 5 per cent. suspension of the washed red cells of the donor are mixed with 0.5 c.c. of 0.85 per cent. sodium chloride solution (control) and another 0.5 c.c. of the suspension of washed red cells from the donor are mixed with 0.5 c.c. of the recipient's serum. The red cells are then examined

microscopically for agglutination. If no agglutination has taken place in any of the mixtures, the blood of the donor and the blood of the recipient are compatible.

8. The formation of rouleaux of red cells is often confused with the formation of masses of agglutinated cells. Inasmuch as rouleaux formation is not significant from the point of view of blood grouping or blood compatibility care must be taken to distinguish between the two phenomena. Rouleaux are readily disassociated by agitation whereas masses of agglutinated cells are permanently adherent.

9. Individuals of the same group differ apparently in the quantity of agglutinin in their sera and in the quantity of agglutinogen in their cells. One sees, therefore, certain instances in which the majority of the red cells are agglutinated and again other instances in which very few red cells are agglutinated. The important observation is not the number of cells agglutinated but the presence or absence of *any* agglutination.

10. Although the group of a normal individual, once established, does not change, an apparent loss of agglutinogen has been observed in cases of long standing anemia of hemolytic type and in other conditions. Moreover, it is possible to immunize animals against red corpuscles of the same group by repeated transfusion (O. H. Robertson and Rous) and the same phenomenon has been observed in man (Kimpton). It is, therefore, necessary to determine the compatibility of the blood of donor and recipient before *each* transfusion.

11. From the point of view of blood incompatibility the danger in transfusion lies in the introduction of red corpuscles that might be agglutinated and hence hemolyzed. It has been said, therefore, that individuals belonging to Group I (Jansky) are universal donors and that individuals belonging to Group IV (Jansky) may receive without harm the blood of any group. The occurrence of severe hemolytic reactions following the use of blood from a donor apparently of Group I (Jansky) for a recipient of another group and following the use of blood from a donor of another group for a recipient apparently of Group IV (Jansky) has resulted in the establishment of the clinical practice of confirming in each case the determination of compatibility of blood specimens by the direct test. It is obvious, however, that time will be saved if the prospective donors are grouped before the direct test of compatibility is made.

12. It has been shown (Ottenberg, vonDungern, Hirschfeld, Buchanan and others) that the isoagglutination group of an individual is an inherited feature following the Mendelian laws and it is possible that blood grouping may turn out to have an important application in medicolegal problems. In the present state of our knowledge, however, it would seem hazardous to settle questions of legitimacy of offspring on the basis of blood group determination. (See the articles by Ottenberg[39] and Buchanan.[40])

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METHODS FOR DEMONSTRATING THE PRESENCE
OF OXIDASE IN LEUCOCYTES

Schultze's Oxidase Reaction.—Many cells possess an oxidizing ferment, which they disclose by forming synthetically naphthol blue when they are treated first with α-naphthol and then with dimethyl-p-phenylendiamin. The method is particularly useful for differentiating myelocytes from cells of the lymphocyte series; the myelocytes, give a positive reaction, while the lymphocytes are negative.

The reaction is carried out as follows (two solutions are required):

1. One gram of α-naphthol is heated to boiling in 100 c.c. of distilled water until it melts and floats in the water. Pure potassium hydrate (about 1 c.c.) is added until all the naphthol is dissolved. The solution appears at first yellow, later yellowish brown.

2. One per cent. aqueous solution of dimethyl-p-phenylendiamin (Merck), made at room temperature and filtered.

Cover-glass preparations fixed in formaldehyde vapor or frozen sections of formaldehyde fixed tissue are placed in solution (1) for a few minutes, and then in solution (2) for a similar length of time; they should be moved gently back and forth in the solutions.

The preparations after being washed in distilled water should be mounted and examined in water or glycerin jelly. The granules, which exhibit the oxidase reaction, are stained deep blue. The preparations are not permanent.

**Graham's Alphanaphthol=pyronin Stain for the Oxi=
dase Granules.[1]—A. Method for Blood Smears.**—1. Allow the smear to dry thoroughly in the air, then fix for one or two minutes in a freshly prepared mixture of 9 parts of 95 per cent. alcohol and 1 part of strong formaldehyde solution.

2. Wash in water and stain for five minutes in a mixture made as follows:

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[1] Graham, G. S., *Journ. Med. Research*, 1916, xxxv, 231–242.

Alphanaphthol (Merck's "Recrystallized" or
Merck's "Reagent"),   1 gm.;
Forty per cent. alcohol,   100 c.c.;
Hydrogen peroxide,   0.2 "

To 2 c.c of this solution add immediately before use one drop of a 2 per cent. aqueous solution of pyronin.

3. Wash off in tap water.

4. Differentiate and remove excess of alphanaphthol by washing in 40 per cent. alcohol for thirty seconds.

5. Wash in water.

6. Counterstain in a 1 per cent. solution of methylene-blue for one-half to one minute.

7. Wash in water, blot and dry.

8. Mount in neutral balsam.

The resulting picture is much like that afforded by the Romanowsky stains, except for the greater prominence of the granules. The neutrophilic granules are usually very abundant, so that the cytoplasmic substance of the cell appears almost completely filled with them. They are somewhat irregular in form and size and are purplish-red in color. Occasional cells show fewer and more faintly stained granules. They may represent old degenerating forms of the cell. The eosinophilic granules are larger, somewhat lighter and more refractile, and have the appearance of spherical bodies with lighter staining centers. The mast-cell granule takes a more basic stain, so that it appears of a deep purple color. Myelocytes have granules of varying number and size. Erythrocytes are greenish-yellow to pink; platelets, blue; nuclei of all cells, blue; cytoplasm, light blue.

The best results are secured with fresh smears or with such as are not over a·few days old. After ten days to two weeks the reacting substances begin to disappear, so that in older specimens many of the granules fail to stain and some cells may appear entirely devoid of them. The eosinophilic granule retains its staining power much longer than the neutrophilic.

B. **Method for Tissues.**—The material must be formalin-fixed and freshly cut frozen sections must be used. After standing twenty-four to forty-eight hours in water the granules may fail to react.

1. Stain rather lightly in alum-hematoxylin. The solution must not be too acid.

2. Wash in water, then for about five minutes in a saturated aqueous solution of lithium carbonate, and return to water for a few minutes.

3. Stain for ten minutes in the same alphanaphthol-pyronin solution used for staining smears.

4. Wash in water, then place for fifteen to twenty minutes in a saturated aqueous solution of lithium carbonate. Wash thoroughly in several changes of water.

5. Differentiate and dehydrate in 80 per cent., followed by 95 per cent., alcohol, transfer to a slide, and clear with xylol by the blotting method.

6. Mount in neutral balsam.

The preparations show an intense red coloration of the granules. Nuclei are greenish-blue to blue.

Graham's Benzidine Stain for the Oxidase Granules.—Keep on hand a small stock bottle of 40 per cent. alcohol containing two-tenths per cent. of hydrogen peroxide. At time of staining saturate the quantity of this fluid required with benzidine. It is very sparingly soluble.

A. **Method for Smears.**—1. Fix fresh smears in a freshly prepared mixture of

95 per cent. alcohol,	9 parts;
Formalin,	1 part.

for one to two minutes.

2. Wash off with tap water.

3. Cover with the benzidine mixture for five to ten minutes.

4. Wash off with tap water.

5. Stain with Loeffler's alkaline methylene-blue solution for thirty seconds.

6. Wash in water, blot and dry.

7. Mount in neutral xylol colophonium or balsam.

The neutrophilic and eosinophilic granules are stained a warm brown. Endothelial leukocytes often contain a few vaguely defined granules which give a positive peroxidase reaction. The nuclei of the leukocytes are dark blue, those of the lymphocytes of a slight purplish tone. The erythrocytes are greenish yellow to greenish blue, the blood platelets blue.

B. **Method for Tissues.**[1]—1. Fix in formaldehyde.

2. Stain freshly cut frozen sections in the above benzidine solution for five minutes.

3. Wash in water.

4. Counterstain rather deeply in alum hematoxylin.

5. Wash in several changes of water.

6. Dehydrate in 80 followed by 95 per cent. alcohol.

7. Clear on slide by the blotting paper-xylol method.

8. Mount in neutral xylol colophonium or balsam.

The stain is permanent.

Goodpasture's Oxidase Stain.

Alcohol 95 per cent.,	100	c.c.;
Basic fuchsin,	0.05	gm.;
Benzidine,	0.05	"
Sodium nitroprusside,	0.05	"
Hydrogen peroxide,	0.5	c.c.

Dissolve sodium nitroprusside in 1 or 2 c.c. of water and add to alcohol. Other ingredients are then added, and shaken until dissolved.

Smears.—Fresh smear dried in air, covered with few drops of reagent; fixed one minute; add equal volume of water and stain three minutes wash in water and blot. Dry and mount in xylol colophonium or balsam.

Tissue.—Frozen sections fixed in formalin. Fix to slides by gelatin or other methods and stain one to three minutes in stock reagent diluted with equal volume of water. Dehydrate in acetone, clear in xylol and mount in xylol colophonium or balsam.

McJunkin's Benzidine Stain.—Embedding.—1. Bits of formalin-fixed tissue 1 mm. thick are placed in 70 per cent. acetone for one hour.

[1] Graham, G. S., *Journ. Med. Res.*, 1918, xxxix, 15–24.

2. Acetone, thirty minutes.

3. Benzol, twenty minutes.

4. Paraffin, twenty minutes.

Staining.—1. Sections 3.5 to 5 microns in thickness, cut in the usual way, are attached to slides with albumin-fixative and allowed to dry overnight at room temperature.

2. Remove paraffin with benzol (twenty seconds) and acetone (ten seconds).

3. Plunge the slide into water for a few seconds, remove the excess of water and apply for five minutes a benzidin solution (25 c.c. of 80 per cent. pure methyl alcohol, 100 mgm. benzidin and 2 drops hydrogen peroxide) diluted at the time of placing it on the preparation with 1 or 2 parts of distilled water. Two parts give less intense staining. The alcoholic benzidin solution is permanent.

4. Plunge into water for five minutes and stain with hematoxylin (Harris) for two minutes.

5. Plunge into water for one minute and stain with eosin (0.1 per cent.) for twenty seconds.

6. Dehydrate with 95 per cent. alcohol (thirty seconds) and absolute alcohol (five seconds).

7. Xylol.

8. Mount in balsam.

The reacting cytoplasm which is arranged in the form of more or less definite granules is colored yellow to brown. The initial color formed is blue, but this soon changes to brown. In sections the cytoplasm overlying the nucleus tends to obscure the nuclear staining, but the chromatin of the nucleus appears not to react.

SEROLOGICAL TECHNIQUE

Widal Test.—This test is used chiefly to determine the presence or absence of specific agglutinating substances in the sera of individuals suffering from disease simulating enteric fever. It is applicable using as an antigen not only B. typhosus but also B. paratyphosus A or B, or type strains of the dysentery bacilli.
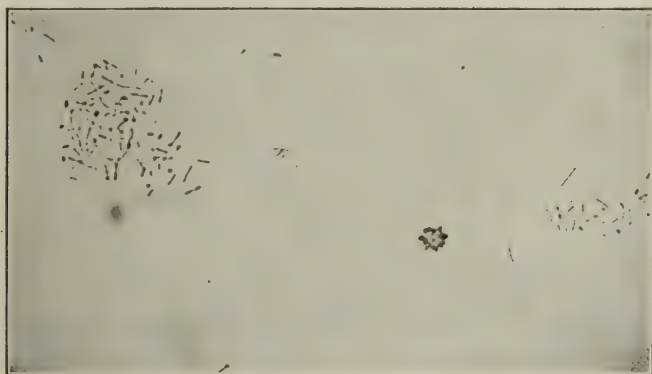
Fig. 147.—Showing the clumping of typhoid bacilli in the serum-reaction. Wet preparation, not stained. At one point a crenated red blood-corpuscle is seen (Wright and Brown).

Microscopical.—On a clean glass slide within three separate spaces not larger than a cover-glass place nine, four, and five platinum loopfuls of an eight to eighteen hour culture of B. typhosus. A salt solution or bouillon suspension of a young agar slant culture may be used, if desired. To the nine loopfuls add one loopful of the serum to be tested. Mix thoroughly and transfer one loopful of the mixture to the four loopfuls of culture. In this way the serum is diluted 1:10 and 1:50. Place clean dry cover-glasses over the two serum culture mixtures and the culture control. Observe under a microscope with the high dry lens after thirty minutes and again after an hour.

Loss of motility and agglutination or clumping of the bacilli in the 1:50 serum dilution mixture constitutes a positive reaction, providing the organisms in the culture control still retain their motility. Loss of motility or agglutination in the 1:10 serum dilution mixture or loss of motility in the 1:50 serum dilution mixture is considered a doubtfully positive reaction. A positive test in the absence of a previous attack of typhoid fever, in the absence of prophylactic antityphoid vaccination and in the presence of disease simulating enteric fever and of two weeks or more duration usually signifies that the disease present is due to typhoid infection. In true cases of typhoid fever positive reactions are obtained but rarely before the end of the second week.

Macroscopical.—In a series of seven *clean* small test-tubes add 0.8 c.c. of sterile physiological salt solution to the first tube, and 0.5 c.c. to all the others. To the first tube add 0 2 c.c. of the serum to be tested. Mix thoroughly and add 0.5 c.c. of the mixture to the second tube. Mix and transfer 0.5 c.c. to the third tube. Continue in this way up to the seventh tube, discarding 0.5 c.c. of the mixture in the sixth tube. Six tubes will then contain 0.5 c.c. of serum dilution and the seventh 0.5 c.c. of salt solution. To all tubes add c.5 c.c. of an eight to eighteen hour bouillon culture of B. typhosus or a salt solution suspension of a young agar culture of B. typhosus. The final serum dilutions are 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. The seventh tube serves as a control. Place in a water bath at 55° C. for one or two hours and read immediately or after placing in an ice-box overnight. It is advisable to run two control series, one with a normal serum and the other with a known high titer antityphoid serum.

The titer of the serum is determined by noting the highest serum dilution in which definite agglutination or clumping occurs. Agglutination should be absent in the salt solution control and in all of the normal serum control series except possibly the first and second and rarely the third tubes. In the antityphoid serum control series the organisms should be agglutinated in all the higher serum dilutions, but agglutination may

be absent in the lower dilutions (pro-agglutinoid zone). In the presence of disease simulating enteric fever and in the absence of a previous attack of typhoid fever and of prophylactic antityphoid vaccination a titer of 1:80 or higher usually indicates typhoid infection. A gradual rise in titer during the second and third week of such a fever is even stronger evidence in favor of typhoid infection.

Wassermann Reaction.—The importance of the so-called Wassermann reaction in syphilis, aside from its scientific interest, lies in the fact that it affords a method by means of which accuracy in diagnosis may be increased, treatment better regulated, and probably a more definite opinion as to prognosis arrived at. The true nature of the reaction is still undetermined. We know that it is a complicated biological reaction, based on the phenomenon of complement fixation. A brief consideration of the theory of this phenomenon will be necessary before stating the method of carrying out the test.

In the first place, a few terms that are constantly being used will be defined:

Complement (*Alexin*).—This is a substance which is found in all fresh sera; its activity is destroyed by exposure to heat at 55° or 56° C. for half an hour. Serum treated in this way is said to be inactivated. The sera of various animals differ in their complementary activity and also in their fixability, which is another characteristic possessed by complement. In selecting a serum for the Wassermann reaction it is best to choose one which has the greatest degree of complementary activity and fixability. It has been found that guinea-pig serum fulfils these demands probably better than the serum of any other species. Anti-complementary action is a property which develops in a serum on standing or which may be present to a certain degree at the time the serum is drawn.

Amboceptor.—This is a specific reaction product, which may be present in any normal serum, and which can be produced in the serum of an animal by repeated injections (immunization) of cells or substances (erythrocytes, serum, egg-albumin, etc.). Amboceptors that are normally present in serum are

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called natural amboceptors; those which are produced as the result of artificial immunization are called immune amboceptors. Amboceptors are classified according to the particular substances employed in their production; for example, hemolytic amboceptors (also called hemolysins) are those that are produced by the injection of foreign red blood-corpuscles into an animal, and bacteriolytic amboceptors (bacteriolysins) are produced by the injection of bacteria. Hemolytic amboceptors are specifically defined by prefixing the term "anti-" to the name of the particular species of corpuscle employed in its production; for instance, when sheep's erythrocytes are the immunizing agent, the amboceptor is designated as an anti-sheep hemolytic amboceptor.

Complement and amboceptor are two of the factors necessary for the production of serum hemolysis. This can be demonstrated by a simple experiment, as follows: immunize a rabbit to human red blood-corpuscles by means of repeated injections, thereby producing in the rabbit serum an anti-human hemolytic amboceptor. If serum from such a rabbit is brought into contact with a suspension of washed human red blood-corpuscles, dissolution of the corpuscles or hemolysis will take place; if, however, the rabbit serum be heated to 56° C. for one-half hour (inactivated), and then corpuscles added, no hemolysis will occur. Finally, if fresh normal serum be added to the latter mixture, hemolysis will go on as before. These three factors which enter into this reaction, namely, the complement, the hemolytic amboceptor, and the red blood-corpuscles, constitute what is called, for the sake of brevity, the hemolytic system.

The function of the amboceptor in the above reaction of hemolysis is to sensitize or prepare the erythrocytes for the action of the complement; then on the addition of the latter hemolysis occurs. Neither amboceptor nor complement acting alone can produce this result. For complete hemolysis a definite ratio must exist between the various factors—amboceptor, complement, and erythrocytes. The requisite strength and proportion of these three can readily be estimated by titration, which will be taken up later.

Antigens and Antibodies.—Antigens are nitrogenous substances which, when injected into a suitable animal, are capable of producing in that animal immune substances called antibodies, the latter thus being specific reaction products. Erythrocytes, bacteria, and serums are examples of antigens. Under antibodies are included hemolytic and bacteriolytic amboceptors, agglutinins, and precipitins. Antibodies are found in the serum of patients following infections with micro-organisms. After typhoid fever, for instance, an antibody is present in the patient's serum as a result of the action of the typhoid bacillus upon the immunizing mechanism of the body.

Generally speaking, it may be stated that antigens and antibodies bear a specific relationship toward one another; for instance, the hemolytic amboceptor produced by injecting a rabbit with sheep's red blood-corpuscles acts with these corpuscles only and with no others. The agglutination of typhoid bacilli by the serum of the typhoid patient is also an example of this connection between antigen and antibody; this fact is made practical use of in the Widal reaction for the determination of the typhoid agglutinin (antibody). The phenomenon of precipitation is another instance of the visible and direct action between antigen and antibody. Both agglutination and precipitation are dual mechanisms requiring no third reagent (complement) to complete the reaction.

Wassermann believed that in syphilis an antibody was developed in the patient's serum through the antigenic action of Treponema pallidum. At present it is generally believed that the substance in positive serum capable of fixing complement is not a true antibody in any sense of the word. However, from a practical standpoint it may be said that it is the presence or absence of this so-called syphilitic anti-body that we seek to demonstrate in the serum diagnosis of the disease.

Complement Fixation.—As stated above, antigen and antibody unite with one another specifically, and furthermore, when united, they acquire the property of fixing or absorbing complement. This fact can be best illustrated by the following example. Take a mixture containing a suspension of

plague bacilli (antigen), inactivated anti-plague serum (anti-body), and fresh normal serum (complement). In the course of several hours at room temperature the antiplague serum unites with the plague bacilli, and this antibody-antigen combination is able to absorb or fix the complement. The process is evidenced by dissolution of the bacilli, and may be hastened by exposure to incubator temperature. If a proper amount of complement has been added, that is, less than the amount capable of being absorbed by the antibody-antigen combination, no hemolysis will occur on the addition of sensitized red blood corpuscles (red blood corpuscles plus their specific hemolytic amboceptor). If heated normal serum is substituted for the heated antiplague serum in the original mixture, hemolysis occurs on the addition of sensitized red blood corpuscles since there is no antibody antigen combination to absorb or fix the complement and the latter is available for the hemolysis of the sensitized cells. This is the well-known phenomenon of complement fixation or deviation of Bordet and Gengou, upon which the Wassermann reaction and its various modifications are based. Theoretically the so-called syphilitic antibody present in a patient's serum when brought into contact with a proper antigen is capable of fixing complement. This reaction is indicated by absence of hemolysis when the other two factors, red cells and amboceptor, of a hemolytic system are added. The five necessary constituents of the reaction are as follows: complement (fresh normal guinea-pig serum), antigen, patient's serum, washed sheep red blood corpuscles, and antisheep hemolytic amboceptor (obtained by immunizing rabbits).

When the reaction was first introduced, it was thought that the antigen used was specific, as it was then made from the liver of a syphilitic fetus. This was the nearest approach obtainable to actual extracts of the causative agent, namely, Treponema pallidum. It has been conclusively proved that this original antigen is not specific, as it has been found that extracts of normal livers, as well as other organs, and also certain lecithin preparations will fix complement in contact with not only leutic sera but also sera from patients infected with leprosy,

yaws, sleeping-sickness, and malaria. The aqueous extract of the liver of a syphilitic fetus, which is used as an antigen in the original Wassermann reaction, is usually not employed on account of its instability, and, incidentally, on account of the frequent difficulty of obtaining syphilitic fetuses. Plain alcoholic extracts of normal organs and of livers and spleens of syphilitic fetuses have been used by many workers in the Wassermann reaction, but at the present time the best antigen seems to be an alcoholic extract of human or bovine heart muscle saturated with cholesterin. On account of its stability for long periods, this antigen is particularly valuable, especially when reactions are done at infrequent intervals.

General.—Many modifications of the original Wassermann technique have been devised and used with success. Various antigens have been employed, as mentioned above; a change in the amounts of the reagents has been recommended; amboceptor in dried form has been tried; other hemolytic systems have been suggested; and so on. It is difficult to say which is the best method. Any method which gives accurate results is satisfactory, and the accuracy of the reaction can be determined only by testing many sera from untreated and treated individuals with definite syphilitic histories. It is possible to standardize the reagents so that a moderate degree of complement fixation is usually a sure indication of syphilis, with the result that the majority of sera from treated cases will give negative reactions. On the other hand the reaction can be adjusted so finely that the majority of sera from treated cases will produce more or less fixation with the result that many sera from unquestionable non-syphilitic individuals will give some degree of fixation. Undoubtedly the ideal adjustment lies in between these two extremes. Consistent results week in and week out are obtained only by the *exact* duplication of the various steps in the technique. This last point cannot be too strongly emphasized. It is recommended that, so far as possible, the test be carried out always by the same individual.

The following technique is that used at the laboratory of the Boston City Hospital for the past three or four years.

Preparation and Standardization of Reagents.—*Glassware.*—The following glassware is required, figured on a basis of 100 sera to be tested:[1] 250 test-tubes (125 × 16 mm.), 125 1.0 c.c. pipettes (graduated to 0.05 c.c.), 10 1.0 c.c. pipettes (graduated to 0.01 c.c.), 6 10 c.c. pipettes (graduated to 0.1 c.c.), 6 150 c.c. bottles or flasks, 100 capillary pipettes, 50 pieces of glass rod about 15 cm. in length, 5 50 c.c. ungraduated centrifuge tubes, and a glass siphon of the proper size to siphon from a 50 c.c. centrifuge tube.

In performing the reaction absolute bacteriological asepsis is not required. All tubes, pipettes, and bottles or flasks should be thoroughly washed in clean cold water, *without soap or chemicals*, and dried in a hot-air sterilizer.

Physiological Salt Solution.—Dissolve 8.5 grams of C. P. sodium chloride in one liter of distilled water, filter and autoclave.

Amboceptor.—Inject a rabbit intravenously or intraperitoneally with 2.0, 3.0, 5.0, and 8.0 c.c. of a 5 per cent. salt solution suspension of carefully washed sheep red blood corpuscles at five or six day intervals. Wait eight days and then remove a few cubic centimeters of blood from an ear vein. Test the serum obtained, and, if the amboceptor unit is greater than 0.001 c.c., repeat the last three injections and retest. Serum with an amboceptor unit less than 0.001 c.c. is satisfactory. The animal should be bled to death aseptically from the carotid artery nine or ten days after the last injection. Collect for several days the serum expressed from the clot, pool, mix, inactivate (56° C. for thirty minutes), and store at ice-box or freezing temperature in sealed glass tubes (about 1.0 c.c. in each tube). The actual sealing is accomplished by drawing up the serum in sterile capillary pipettes made from small glass tubing and by sealing first at the tip and then at the open end a short distance above the level of the serum.

The amboceptor unit is the smallest amount of amboceptor which will cause complete hemolysis of 0.5 c.c. of a 5.0 per cent.

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[1] If the maximum number of sera to be tested is larger or smaller than 100, the number of test-tubes, 1.0 c.c. pipettes (graduated to .05 c.c.), capillary pipettes, and rods should be varied proportionately:

cellular suspension in the presence of an excess of complement. To a series of test-tubes add 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3. 0.2, and 0.1, c.c. of a 1:1000 dilution of amboceptor in salt solution, add 0.1 c.c. of undiluted complement or 1.0 c.c. of 1:10 complement, add 0.5 c.c. of a 5.0 per cent. cellular suspension, and add salt solution to a total volume of 2.5 c.c. in each tube. Mix by shaking vigorously and place in a water bath at 37° C. for fifteen minutes. Suppose that the smallest amount of amboceptor causing complete hemolysis is 0.3 c.c., which is equivalent to a 1:3333 dilution, then one amboceptor unit is contained in 1.0 c.c. of a 1:3333 dilution. Since two amboceptor units contained in a total volume of 0.5 c.c. are used in the actual test, the desired dilution is $\dfrac{2 \times 2}{3333}$ = approximately 1:800.

Each fresh ampoule of amboceptor is diluted 1:100 with sterile salt solution and, if kept in a tightly corked bottle at ice-box or freezing temperature, will retain its potency for several weeks. The amboceptor unit should be determined for each new ampoule, and the actual dilution to be used for the test should be figured accordingly. In order to be sure that no deterioration has occurred the final amboceptor dilution should be checked each day before a test at the same time as the determination of the complement unit. For this purpose add to a series of tubes 0.35, 0.30, 0.25, and 0.20 of the final amboceptor dilution, and to each add 1.0 c.c. 1:10 complement, 0.5 c.c. of 5.0 per cent. cellular suspension, and salt solution to a total volume of 2.5 c.c. Mix by shaking vigorously and place in a water bath at 37° C. for fifteen minutes. Complete hemolysis should occur in the 0.35 and 0.30 c.c. tubes and complete or nearly complete hemolysis in the 0.25 c.c. tube.

At this point it is well to emphasize the importance of vigorously shaking the mixtures in the tubes after each addition both during standardization of the reagents and during the test. Failure to do so will often lead to irregular results. For example, we know that red cells are capable of absorbing fifteen or twenty times as much amboceptor as is necessary for hemolysis on the addition of complement. Let us call the amount of amboceptor

necessary for the hemolysis of one red cell an amboceptor cellular unit and let us suppose that to a solution of amboceptor containing 100 amboceptor cellular units we add 100 red cells. If each red cell united with one amboceptor cellular unit, hemolysis of all cells would occur on the addition of complement. However, when the cellular suspension is pipetted into the test-tube containing the amboceptor, it follows down the wall of the tube to the bottom forming a more or less regularly defined bottom layer. It is conceivable that ten cells in the upper portion of this layer, which is in direct contact with the amboceptor, will absorb, let us say, seven cellular units each of amboceptor. On subsequent shaking this would leave only 30 cellular amboceptor units to be divided among the remaining 90 cells, and no matter how much complement is added these 90 cells would never undergo hemolysis. Of course this is a purely hypothetical case, but it serves to furnish an exaggerated idea of the unbalanced conditions which may occur in the absence of proper mixing.

Complement.—Remove sufficient blood from four or five adult guinea-pigs by intracardiac puncture to give a total volume of 25 to 30 c.c. After each bleeding the blood should be discharged into a porcelain evaporating dish (150 cm. in diameter) and defibrinated by beating with a fork. For the bleeding, sterilization of the syringe and needle is not necessary; they should be rinsed out with sterile salt solution before and after each bleeding. By keeping eight or ten sets of four or five guinea pigs and using each set in rotation blood can be obtained two or three times a week without any ill effect on the guinea-pigs.

Pour the defibrinated blood into a 50 c.c. glass centrifuge tube and centrifuge at high speed for ten or fifteen minutes. With a capillary pipette transfer the clear supernatant serum to a sterile test-tube. The serum should be obtained the afternoon previous to the day of the test and should be stored overnight in the ice-box.

For the actual test the complement (serum) is diluted (1 : 10) by adding nine parts of sterile salt solution. Complement

deteriorates quite rapidly at room temperature when diluted and for this reason it is advisable to keep the bottle or flask containing the diluted complement immersed in ice water.

The complement unit is the smallest amount of complement which will cause complete hemolysis of 0.5 c.c. of a 5.0 per cent. cellular suspension in the presence of two amboceptor units. This unit must be determined each day shortly before the test is carried out. To a series of test-tubes add 0.2, 0.3, 0.4, 0.5, and 0.6 c.c. of a 1:20 dilution of complement, add 1.0 c.c. of a mixture of equal parts of a 5.0 per cent. cellular suspension and an amboceptor dilution of such strength that each 0.5 c.c. contains two amboceptor units, and add salt solution to a total volume of 2.5 c.c. Mix by shaking vigorously and place in a water bath at 37° C. for fifteen minutes. Having determined the smallest amount of 1:20 complement causing complete hemolysis, this same amount of the 1:10 dilution is the quantity to be used in the actual test, *i.e.* two complement units.

Antigen.—Free fresh human or bovine heart muscle from the larger blood vessels and excessive fat, pass through a meat grinder, and add nine times its weight in cubic centimeters of 95 per cent. or absolute alcohol. Place in an incubator at 37.5° C. for nine or ten days, shaking frequently. Filter and add 0.4 per cent. of cholesterin. Replace in the incubator for four or five days, shaking frequently. If contained in a well-stoppered bottle such an antigen will keep indefinitely at room temperature. Before use each antigen must be standardized and also tested for its anticomplementary and hemolytic action.

If an antigen of known potency is available a new antigen is most accurately standardized as follows: determine the smallest amounts of several known positive sera which in the presence of the usual amount of the old antigen will produce sufficient complement fixation completely to inhibit hemolysis. With these minimum amounts repeat the tests using in place of the old antigen 0.1 c.c. of 1:5, 1:10, 1:15, 1:20, and 1:25 salt solution dilutions of the new antigen. An average of the smallest amounts of the new antigen required completely to

inhibit hemolysis determines the desired antigen dilution. This dilution should be checked by comparing the results obtained, on numerous positive and negative sera, with those obtained with the old antigen on the same sera. As a rule the 1:15 or 1:20 dilution of a cholesternized antigen proves to be the correct dilution for use.

In the absence of an antigen of known potency a new antigen can be standardized only by carrying out routine tests on many sera from clinically positive and negative cases using varying dilutions of the antigen. A satisfactory antigen dilution should bring about in the majority of instances complete inhibition of hemolysis with sera from cases of untreated syphilis of moderate or long duration, complete or partial inhibition or complete hemolysis with sera from cases of treated syphilis, and complete hemolysis with sera from non-syphilitic cases.

In regard to the anticomplementary and hemolytic actions, an antigen is satisfactory if 0.4 c.c., *i.e.* four times the amount used in the routine test, does not inhibit hemolysis with negative sera in actual tests and does not produce any hemolysis in a mixture containing 0.5 c.c. of 5.0 per cent. cellular suspension and 1.6 c.c. salt solution after thirty minutes in a water bath at 37° C.

In making the antigen dilution for routine tests great care should be taken to prepare the dilution in precisely the same way each time. If the salt solution is added to the antigen one time and the antigen to the salt solution the next, or if the salt solution is added rapidly to the antigen one time and drop by drop the next, discordant results will be obtained. It makes little or no difference just how the dilution is made so long as the method is not altered.

Cellular Suspension.—Strain about 10 c.c. of sterile[1] defibrinated sheep blood through several thicknesses of sterile gauze into a 50 c.c. centrifuge tube. With a wax pencil place a mark on the outside of the tube to indicate the volume. Centrifuge

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[1] Absolute asepsis is not necessary in collecting and handling the blood unless it is to be kept for several days before use.

and wash with sterile salt solution at least three times or until the supernatant fluid does not contain even a trace of hemoglobin. The supernatant fluid is most conveniently removed by means of a small glass syphon. After the final washing syphon off the supernatant fluid and make up to the original volume with salt solution. The washing of the corpuscles can be carried out on the afternoon preceding the day of the test. The cells should be stored overnight in the ice-box.

For the actual test a 5.0 per cent. suspension of the cells, made up to their original volume, is used. This corresponds roughly to the 2.5 per cent. suspension of "packed" cells used in many laboratories.

Patients' Sera or Spinal Fluids.—The bloods to be tested are brought to the laboratory in small test-tubes (100 × 10 mm.). Frequently the expressed serum can be removed directly by means of capillary pipettes. Usually the clot has to be freed or broken up by means of glass rods. After centrifuging, the clear serum is pipetted off. All badly hemolyzed sera are discarded and a report of "Hemolyzed-unsatisfactory" is returned. The centrifuge cups intended for 50-c.c. tubes will each hold four of the small test-tubes. This separation of the sera is carried on the afternoon before the test.

The morning of the test all the sera are inactivated by heating in a water bath at 56° C. for thirty minutes. This inactivation destroys whatever complement is present in the sera. For the test 0.1 c.c. of each serum is used, and in addition 0.3 c.c. of each serum with which hemolysis is inhibited..

Spinal fluids which are contaminated or which contain an appreciable amount of exudate are usually discarded. For the test 1.0 c.c. of each spinal fluid is used, and in addition 3.0 c.c. of each spinal fluid with which hemolysis is inhibited. Since the spinal fluid does not contain complement, inactivation is unnecessary. Fluids containing any appreciable amount of blood should be centrifuged to remove the corpuscles.

The Test.—In order to explain the various steps as clearly as possible they will be given in exact chronological order. We will assume that there are 100 non-hemolyzed sera and five

satisfactory spinal fluids to be tested, that the amboceptor is of such strength that two units are contained in 0.5 c.c. of a 1:800 dilution and a stock 1:100 dilution is on hand, that the proper dilutions of the cholesterinized antigens *A* and *B* are 1:15 and 1:20 respectively, that there are on hand two positive and two negative sera carried over from the previous test, that some defibrinated sheep blood has been obtained, and that there is sufficient sterile salt solution.

Afternoon before the Test.—1. Bleed guinea-pigs, defibrinate the blood (25–30 c.c.), centrifuge, and pipette away the serum (slightly over 11 c.c.). Store at ice-box temperature overnight.

2. Wash about 10 c.c. of the defibrinated sheep blood three or four times with sterile salt solution, finally making up to the original volume. Store at ice-box temperature overnight.

3. Separate the patients' sera. Discard all badly hemolyzed sera and all badly contaminated or purulent spinal fluids. If necessary, centrifuge the spinal fluids to remove red blood corpuscles. Store the sera and spinal fluids at ice-box temperature overnight.

Day of the Test.—1. Inactivate the patients' sera by heating at 56° C. in a water bath for thirty minutes.

2. To 11 c.c. guinea-pig serum add 99 c.c. salt solution in a bottle or flask. Mix thoroughly and place in an ice water bath. This makes a 1:10 dilution of complement.

3. To 15 c.c. of the 1:100 dilution of amboceptor in a bottle or flask add 105 c.c. salt solution. Mix thoroughly. This makes a 1:800 dilution of amboceptor.

4. To 0.9 c.c. of cholesterinized antigen *A* contained in a test-tube add 12.6 c.c. salt solution. Mix thoroughly. This makes a 1:15 dilution of antigen *A*.

5. To 6.0 c.c. of washed sheep corpuscles in a bottle or flask add 114 c.c. of salt solution. Mix thoroughly. This makes a 5.0 per cent. cellular suspension.

6. Make a 1:20 complement dilution by adding 1.5 c.c. salt solution to 1.5 c.c. 1:10 complement. Mix thoroughly.

7. To a series of four tubes add 1.0 c.c. 1:10 complement, 0.35, 0.30, 0.25 and 0.20 c.c. 1:800 amboceptor, 0.5 c.c. 5.0 per

cent. cellular suspension, and salt solution to a volume of 2.5 c.c. To a series of five tubes add 0.6, 0.5, 0.4, 0.3 and 0.2 c.c. 1:20 complement, 0.5 c.c. 1:800 amboceptor, 0.5 c.c. 5.0 per cent. cellular suspension, and salt solution to a volume of 2.5 c.c. To another tube add 0.4 c.c. 1:15 antigen A, 0.5 c.c. 5.0 per cent. cellular suspension, and 1.6 c.c. salt solution. To another tube add 0.5 c.c. 5.0 per cent. cellular suspension and 2.0 c.c. salt solution. Shake all sets of tubes vigorously after each addition. Place all tubes in a water bath at 37° C. for fifteen minutes. In the first series the tubes containing 0.35 and 0.30 c.c. of amboceptor should show complete hemolysis, and the 0.25 c.c. tube may or may not show complete hemolysis. If the amboceptor unit is higher or lower, weaken or strengthen respectively the cellular suspension and repeat the titration. If the cellular suspension is weakened or strengthened all the tests must be repeated. From the second series of tubes determine the complement unit.

The first and second single control tubes should both show no hemolysis, indicating that the antigen is non-hemolytic and the salt solution isotonic respectively. We will assume that the amboceptor titration is satisfactory, that the complement unit is 0.5 c.c., and that the two single control tubes show no hemolysis.

8. To 11.0 c.c. 1:15 antigen A contained in a bottle or flask add 55 c.c. 1:10 complement and 88 c.c. salt solution. Mix thoroughly. In this mixture 1.4 c.c. contains 0.1 c.c. of 1:15 antigen A and 0.5 c.c. 1:10 complement.

9. To a series of 104 tubes add 0.1 c.c. of patients' sera and of the control sera and then add 1.4 c.c. of the preceding antigen-complement mixture. To a series of 5 tubes add 1.0 c.c. of the spinal fluids, 0.1 c.c. 1:15 antigen A and 0.5 c.c. 1:10 complement. To a single tube add 0.1 c.c. negative control serum, 0.4 c.c. 1:15 antigen A, 0.5 c.c. 1:10 complement, and 0.5 c.c. salt solution. Shake all sets vigorously after each fresh addition. Place all tubes in a water bath at 37° C. for one hour. In the meantime to 55 c.c. 5.0 per cent. cellular suspension contained in a bottle or flask add 55 c.c. 1:800 amboceptor.

Mix thoroughly and place in the water bath at 37° C. for thirty minutes. At the end of the one hour heating period add to each of the tubes 1.0 c.c. of the sensitized cellular suspension. Shake vigorously and place in the water bath at 37° C. for thirty minutes. Pick out the sera and fluids in which there is inhibition of hemolysis, making notations as to whether the inhibition is complete $(+++)$, moderate $(++)$, slight $(+)$, or questionable $(\pm)$. The single control tube containing 0.4 c.c. 1:15 antigen A should show complete hemolysis, indicating that the antigen is not anticomplementary with the quantity used. We will assume that there is inhibition of hemolysis with ten of the sera and two of the spinal fluids, not including the two positive control sera.

10. To 0.2 c.c. antigen B contained in a test-tube add 3.8 c.c. salt solution. Mix thoroughly. This makes a 1:20 dilution of antigen B.

11. Set up four rows (first, second, third and fourth) of 16 tubes each. To the second and fourth rows add 0.1 c.c. of the ten positive and four control sera and 1.0 c.c. of the two positive spinal fluids. To the first and third rows add 0.05 c.c. of the same sera and 0.5 c.c. of the same spinal fluids. To the first row add 0.05 c.c. 1:15 antigen A, and to the second and third rows add 0.1 and 0.05 c.c. 1:20 antigen B respectively. To all the tubes add 0.5 1:10 complement. To the tubes in the second row add 0.8 c.c. salt solution and in the first, third and fourth rows 0.9 c.c., and to the spinal fluid tubes in the first and third rows add 0.45 c.c. salt solution. To a single tube add 0.1 c.c. negative control serum, 0.4 c.c. 1:20 antigen B, 0.5 c.c. 1:10 complement, and 0.5 c.c. salt solution. Shake all tubes vigorously after each addition. Place in a water bath at 37° C. for one hour. In the meantime to 30 c.c. 5.0 per cent. cellular suspension contained in a bottle or flask add 30 c.c. 1:800 amboceptor. Mix thoroughly and place in the water bath at 37° C. for thirty minutes. At the end of the one hour heating period add to each of the tubes 1.0 c.c. of the sensitized cellular suspension. Shake vigorously and place in the water bath at 37° C. for thirty minutes. Note the absence

or degree of inhibition of hemolysis in all tubes. The single control tube containing 0.4 c.c. 1:20 antigen *B* should show complete hemolysis, indicating that the antigen is not anti-complementary with the quantity used.

12. Pick out two positive and two negative sera to serve as controls for the next test. These should be kept in the ice-box. Replace the 1:100 amboceptor in the ice-box.

13. Wash all the glassware carefully with clean cold water. It is advisable to allow the pipettes to soak overnight in water in order to soften up and dissolve any serum which may have dried on the inner walls.

Interpretation of Results.—All sera and spinal fluids showing complete hemolysis with antigen *A* are reported "Negative." All sera and spinal fluids showing inhibition of hemolysis with antigens *A* and *B* but at the same time inhibition in the anticomplementary control (fourth row of second test) are reported "Anticomplementary." The other sera and spinal fluids are potentially positive and depending upon the degree of inhibition are reported according to the following scheme:

Full quantity serum (or fluid) and antigen	Half quantity serum (or fluid) and antigen[1]	Report
+++	+++, ++, + or ±	Positive
++	+	Positive
++	± or o	Doubtful
+	± or o	Doubtful
±	o	Probably negative

[1] This is a modification suggested by Citron.

The Wassermann reaction is not absolutely specific for syphilis.

A positive reaction, not due to syphilis, is frequently obtained in yaws, trypanosomiasis, and leprosy, and according to some authorities may also be obtained in rare instances in other conditions, such as scarlet fever, malaria, lobar pneu-

monia, diabetes mellitus, and immediately following ether anesthesia.

A negative reaction is obtained in many cases of syphilis during treatment with salvarsan or mercury, and may occur in alcoholics.

Antisyphilitic treatment must be interrupted for two months before a negative Wassermann reaction can be considered to have any diagnostic value.

Wassermann Reaction—Noguchi Modification.—Probably the most widely used radical modification of the original Wassermann technique is that devised by Noguchi. An anti-human hemolytic system is used, as Noguchi maintains that, owing to the presence in human serum of varying amounts of natural antisheep amboceptor, many positive reactions are rendered negative in the regular Wassermann test due to the increase in the total amount of antisheep amboceptor and the consequent disturbance of the proper proportion between amboceptor and complement. A further advantage lies in the fact that the method may be used conveniently by laboratories where fresh sheep corpuscles are not readily available. As an antigen Noguchi recommends a methyl alcoholic solution of the acetone insoluble fraction of an alcoholic normal organ (heart, liver, or kidney) extract. It has been found that such antigens give fewer false positives than the plain or cholesterinized alcoholic extracts; but, they possess the disadvantage of being relatively unstable. For the details of this method the reader is referred to Noguchi, "Serum Diagnosis of Syphilis," Lippincott, 1910.

A Modification of the Wassermann Reaction (J. H. Wright).—This modification employs as complement and hemolytic amboceptor unheated human serum or a mixture of sera which, on the day of use, have been found capable of hemolyzing a certain amount of sheep corpuscles after contact with a certain amount of antigen.

This preliminary procedure of the method may be described as follows: Select several fresh unheated sera which are probably not syphilitic. Place 0.2 c.c. of each serum in test-

tubes, add to each tube 0.2 c.c. of cholesterinized antigen suspension as in the Wassermann reaction and put the tubes in the water bath at 37° C.; after fifteen minutes add 0.5 c.c. of the sheep blood corpuscle suspension of the Wassermann reaction, diluted with four volumes of normal salt solution, to each tube, and keep in the water bath fifteen minutes more. Then note the tubes in which complete hemolysis has occurred. Mix or pool the corresponding serums which have thus shown themselves to be hemolytic, and titrate the mixture, to determine its minimum dose as hemolyzing reagent in the tests described below. This titration is made as follows: In test-tubes place respectively 0.14, 0.13, 0.12, 0.11, 0.10 and 0.09 c.c. of the pooled serums and in each tube 0.2 c.c. of the antigen suspension described above; then put the tubes in the water bath at 37° C. After fifteen minutes add to each tube 0.5 c.c. of the diluted sheep blood corpuscle suspension described above and keep the tubes in the water bath for fifteen minutes more. Then note the least amount of the pooled serums which has produced complete hemolysis in the tubes. This, or the next greater amount of the pooled serums, is the "dose" to be used in the test.

A number of serums should be tested at the same time, and the tests are carried out with serums *after they have been inactivated* as follows:

Place in test-tubes 0.2 c.c. of each serum, add to each tube 0.2 c.c. of the antigen suspension and the titrated dose of the pooled hemolytic serums. Heat the tubes in the water bath at 37° C. for thirty minutes, after which time add 0.5 c.c. of the diluted blood corpuscle suspension and keep the tubes in the bath for thirty minutes longer. Then examine the tubes for inhibition and degree of hemolysis, as in the Wassermann reaction.

Next test serums, which have shown inhibition of hemolysis in the tubes, for anticomplementary property by placing in a test-tube 0.2 c.c. of the serum, the titrated dose of the pooled hemolytic serums, and 0.5 c.c. of the diluted blood corpuscle suspension, in the order named; and heat in the water bath at

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37° C. If hemolysis is incomplete or lacking after thirty minutes, the serum is "anticomplementary" and unsuitable for the test. Complete hemolysis shows that the inhibition of hemolysis in the previous tube was due to fixation of complement by the serum and that the serum has therefore given a positive reaction.

Comparative tests have shown a very close agreement in results with the regular Wassermann reaction.

The principles upon which this modification is based were first called to attention by the observations of C. J. Bartlett and A. L. O'Shansky.[1]

The Complement Fixation Test in Gonorrheal Infec=tions.—The method and technique are essentially the same as that of the Wassermann test, except that the antigen is different.

Preparation of the Antigen.—The growth from twenty-four-hour cultures of the gonococcus on hydrocele agar slants is scraped off with a platinum loop and suspended in a 0.5 per cent. solution of carbolic acid in distilled water. The proportion of cocci to fluid should be the entire surface growth on the ordinary slant culture in a three-quarter-inch test-tube to about 5 c.c. of the fluid. The suspension is to be kept at room temperature and to be shaken from time to time for a week, after which it should be heated to 56° C. for an hour and centrifugalized. To the supernatant fluid is then added sufficient 9 per cent. sodium chlorid solution to make its content in that salt 0.9 per cent. This is the antigen. Every time it is used it must be titrated to determine its anticomplementary dose, and one-half of the largest amount which does not inhibit hemolysis is to be used in the test. The addition to the antigen of one drop of a saturated alcoholic solution of cholesterin for each cubic centimeter of antigen just before use may be found to increase its fixing power.

The suspension of the cocci in the carbolic acid water retains its fixing power for months, and it may be kept on hand

[1] Bartlett, C. J. and O'Shansky, A. L., *Jour. Lab. and Clin. Med.*, 1917, iii, 118.

in quantities as a stock from which, as needed, small quantities may be taken, centrifugalized, and the proper amount of sodium chlorid added.

Considerable variation in the fixing power of different antigens is observed, and it is advisable to use two or three antigens from different strains of gonococci in each test or a mixture of antigens from several strains.

The Complement Fixation Test in Echinococcus Infection.—The method and technique are the same as those for the Wassermann test, except that the cyst fluid, or an alcoholic extract of the cyst walls, is used as the antigen. The cyst fluid should be clear, and to it should be added carbolic acid in the proportion of 0.5 per cent. to preserve it.

The extract of the cyst walls is prepared by grinding them up with sand and extracting the mass with absolute alcohol, in the proportion of about one part cyst substance to five of alcohol, for several days.

The anticomplementary dose of the fluid or of the extract, the latter diluted 1 to 4 with normal salt solution, is to be determined by titration, and one-fourth of this quantity is to be used in the test.

These antigens are said to keep for months.

Complement Fixation Test in Glanders.—This test is extremely valuable for the diagnosis of glanders in humans and horses. The technique is the same as that of the gonorrheal complement fixation test with the exception of the preparation of the antigen. Stock cultures of the organisms are grown on 1.6 per cent. glycerin potato agar and transplants are made to neutral veal pepton agar. After twenty-four hours the growth is washed off with sterile distilled water and sterilized at 80° C. for four hours. The suspension is filtered through a porcelain candle and reheated at 80° C. for one hour.

Precipitation Test for Syphilis.—The importance of the precipitation test in the diagnosis of syphilis was first brought forth by Meinicke[1] and later the technique was improved and

[1] Meinicke, *Berl. klin. Wochenschr.*, 1917, liv, 613.

-implified by Sachs and Georgi.[1] The Sachs-Georgi test consists in bringing together diluted heated serum and a proper alcoholic antigen dilution. The mixtures are incubated for two twenty-hour periods, the first at $37.5°$ C. and the second at ice box temperature. A positive reaction is evidenced by the appearance of a precipitate. Sachs and Georgi claimed an accuracy at least equal to that of the Wassermann test. During the last five years many thousands of parallel tests have been carried out both in this country and abroad, and their claim has been virtually confirmed. The results obtained with the precipitation test agree in 90 to 95 per cent. of the cases with those obtained with the Wassermann test, and it must be acknowledged that a great many of the disagreements are cases of treated syphilis with the sera of which the precipitation test gives positive and the Wassermann test negative reactions.

Kahn[2] has devised a modification in which the most objectionable feature of the Sachs-Georgi test, the two twenty-hour incubation periods, has been eliminated. The method is so simple and easy to carry out that it is given in detail in spite of the fact that, for the present at least, it should not be employed without a parallel Wassermann test. Because of its simplicity both in regard to reagents and to technique the method lends itself readily to standardization, thereby suggesting the possibility of a standard serological test for syphilis, an ideal practically impossible to attain with the Wassermann test.

Antigen.—Free fresh beef heart from excessive fat, fiber and blood vessels and pass through a meat grinder several times. Spread out in a thin layer and dry in the open air by means of an electric fan. Break into small pieces and pass through a coffee grinder three times. Place 50 grams in a 500 c.c. Erlenmeyer flask and add sufficient ether to cover the material with an inch of fluid. Place in the ice-box. On the next day pour off the ether and replace with fresh ether. Repeat for three days.

[1] Sachs and Georgi, *Med. Klinik.*, 1918, xiv, 805.

[2] Kahn, *Arch. Dermat. and Syph.*, 1922, v, 570 and 734; 1922, vi, 332.

Filter off the ether and allow the material to dry at room temperature until no odor of ether is detectable. To 20 grams of this material add 100 c.c. absolute alcohol. Extract for nine days in the ice-box and one day at room temperature. Filter. Divide the filtrate in approximately two equal portions and to one add 0.4 per cent. cholesterin. The former constitutes the alcoholic antigen and the latter the cholesterinized antigen.

In the actual test the alcoholic antigen is diluted with two times its volume of 0.85 per cent. salt solution and the cholesterinized antigen with three times its volume of salt solution. The dilutions should be accomplished as rapidly as possible. The cholesterinized antigen is relatively unstable at low temperatures and should be kept in an incubator (37.5° C.) when not in use.

The Test.—Measure out 0.3 c.c. of clear inactivated serum into two small test-tubes (100 × 10 mm.). To one add 0.05 c.c. of cholesterinized antigen and to the other the same amount of alcoholic antigen. Shake all tubes vigorously for two or three minutes, and place in an incubator for eighteen hours at 37.5° C. The incubation period can be shortened to six or eight hours by using only a cholesterinized antigen and shaking the tubes for at least one minute at the end of each hour period of incubation.

About 80 per cent. of all sera which give complete inhibition of hemolysis in the Wassermann test will show in the cholesterinized antigen tubes an immediate precipitation after the preliminary shaking following the addition of antigen. This spontaneous reaction also occurs in the alcoholic antigen tubes but in a smaller percentage. Readings are made on all tubes after the eighteen-hour incubation period. The tubes should not be shaken before the readings are made. The $++++$ sera show one or several large clumps, the $+++$ sera a moderate number or large flocculi or granules, the $++$ sera smaller granules but large enough to be unmistakable, and the $+$ and $\pm$ sera definite precipitates which require more or less ideal conditions of illumination to be readily recognized.

EXAMINATION OF CEREBROSPINAL FLUID[1]

While the cerebrospinal fluid is contained in the anatomically continuous spaces of the cerebral ventricles and the subarachnoid spaces of the brain and spinal cord, we should distinguish ventricular fluid from subarachnoid fluid, because under normal conditions these two fluids are somewhat different, and especially because under pathological conditions, the fluids from these loci may be grossly unlike.

An understanding of our present knowledge of the production and flow of cerebrospinal fluid is necessary for an estimation of the significance of changes found in it in different places. It is generally accepted that the main sources of production of the fluid are the choroid plexuses of the ventricles, but it is also probable that a certain amount of it comes from the perivascular spaces of the cerebrum. Fluids from these sources unite in the cisterna magna, which may be considered as the center for the distribution of the combined ventricular-subarachnoid fluids forward over the brain base, and downward over the spinal cord. It is obvious that methods which will allow us to examine the fluid from as many loci as possible are likely to yield information of value; and this is especially so when, from pathological processes, obstruction has interrupted the normal continuity of flow, and caused isolated compartments of fluid. While the determination of "block" in the ventriculo-subarachnoid system is largely a matter of clinical observation with the aid of dyes and manometric studies, it may also be indicated by great variation in the pathological findings of fluids from different loci.

Technique.—As we are not able to get access safely to the cerebrospinal fluid in all regions where it circulates, we must rely, for the most part, on the information obtainable from tapping the most distal portion of the spinal subarachnoid space. This is done by lumbar puncture, a procedure which may be carried out between any pair of lumbar vertebræ.

[1] This section has been prepared by Dr. James B. Ayer and Miss Jessie R. Cockrill.

While in most cases the fluid obtained gives a fair idea of the pathological process in unobstructed channels of the fluid, in conditions where blocking has occurred other methods of approach have proved useful. Of these exceptional methods, two have proved their safety and value in appropriate cases, namely, puncture of the cisterna magna[1] and puncture of the lateral ventricle. It is needless to point out that these methods are potentially dangerous, and should be used only by skillful operators.

Normal Cerebrospinal Fluid.—The fluid obtained from lumbar puncture is water clear and limpid; its specific gravity is 1.006 to 1.008. It contains a trace of albumin, but no globulin, and no fibrin. Decomposition products of protein may be present in small amount. Sugar is present normally, approximately 0.05 per cent. Inorganic salts, particularly sodium chloride, are present in small amounts, also potassium, calcium and magnesium.

The fluid from the cisterna magna and the ventricles, although insufficiently studied, differs from that of the lumbar subarachnoid space. The most conspicuous difference is its lower protein content, especially in the case of the ventricular fluid.

Tests and Observations on the Cerebrospinal Fluid.— The more important are the following:

1. Pressure estimation.
2. Gross appearances of the fluid.
3. Tests for protein and globulin.
4. Cell examination.
5. Wassermann test.
6. Colloidal chemical tests.
7. Sugar quantitation.
8. Bacteriologic examination.

While every fluid need not be subjected to all of these, it is usually found that only by a correlation of findings can information of value be obtained. For example: a high protein fluid with accompanying high cell count indicates a meningeal

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[1] Puncture of the Cisterna Magna, J. B. Ayer, *Arch. Neurol and Psychiat.*, 1920, iv, 529.

reaction, while a fluid containing the same quantity of protein, but without cell increase, suggests a transudative source for the protein, and may be indicative of spinal cord tumor. Our present knowledge of the various tests requires that all of those mentioned should be made routinely on a given fluid, with the exception of sugar determination and bacteriological examination.

1. **Pressure Estimation.**—This is inaccurate if judged by the the rapidity of outflow from the needle, because the bore of the needle and obstructions in it control the flow. To measure pressure of the fluid two types of manometers are available and each has its uses. When high pressures are expected the mercury manometer[1] is most convenient, especially if it is to be used in connection with estimation of ventricular pressure, during operation on the head. For most work the water manometer, with a bore of 2 mm.[2] is preferred, because visible oscillations of pulse and respiration, and slight changes in pressure are more readily observed. The following pressure observations with the patient lying on one side at rest and with unobstructed breathing, should be carried out routinely: The initial pressure to which the fluid rises in the manometer should be measured, the rapidity of fall in pressure on withdrawal of fluid noted, and the effect of compression of the jugular veins on the pressure determined. Normal pressure readings are seldom over 250 mm. (aqueous); above this intracranial pathology is strongly suggested. The fluid pressure normally drops about 50 mm. on withdrawal of 10 c.c., which is an amount of fluid ample for all tests. An excessive drop suggests that a small reservoir of fluid has been tapped; a slight drop suggests hydrocephalus. Temporary elevation of intracranial pressure by means of jugular compression normally produces a prompt and considerable rise in the lumbar manometer; absence of a rise indicates spinal subarachnoid block. It is sufficient here merely to call attention to the possibilities of further dynamic studies

[1] Fleischel, made by Becton-Dickinson Co., Rutherford, N. J.
[2] A special form is made by Codman and Shurtleff, 120 Boylston St., Boston, Mass.

which may be carried out by means of simultaneous combined punctures.

2. **Gross Appearances of the Fluid.**—The fluid should be collected in at least two clean sterile tubes, and if turbid, in three tubes. This serves not only as a precaution against loss by breakage, but, in the case of a blood-tinged fluid, to distinguish between a "bloody fluid" and a "bloody tap." In the former case the three tubes will look alike, in the latter, the first tube will contain more blood than the third.

Turbidity is most commonly due to blood. It may also be due to cells and to bacteria. Fluids turbid from bacteria, may be white, gray, yellow, brown or green. Clear fluids may be yellow from dissolved blood pigment, varying from a deep orange to the palest lemon tint, recognized only when compared with water. Usually yellow tinted fluids are associated with two types of pathological process: Either the fluid has picked up blood pigment from a hemorrhage somewhere in the brain or spinal cord, or else it occurs in connection with a spinal cord tumor. In the latter case, excessive protein content is also present, and at times immediate clotting "en masse," giving the so-called "Froin syndrome." Some fluids clot on standing. A significant and almost constant occurrence in the fluid from tuberculous meningitis is the formation of a very fine web-like clot, attached to the surface and extending downward to the bottom of the tube, which appears after a number of hours.

3. **Tests for Protein and Globulin.**—Perhaps the most important single test performed on the cerebrospinal fluid is the test for coagulable protein. Protein increase should not be looked upon as in itself diagnostic, for it is a non-differential reaction, its derivation being exudative and transudative in character. The normal protein content of the fluid is probably 20 to 40 mg. per 100 c.c. Slight protein increase is found in degenerative diseases of the nervous system, commonly 60–80 mgs. Inflammations of the nervous system or meninges are usually observed with greater amounts; in tuberculous meningitis 200 to 300 mgs. is to be expected, and, in acute meningitis, 400 to

500 mg. The highest figures are found in connection with tumors of the spinal cord, where, in fluids showing the Froin syndrome, the quantity may be 2 to 3 grams per 100 c.c.

(A) *The Alcohol Test for Total Protein.*—Mix 1 part of the fluid with 3 parts of alcohol 95 per cent. Normally a faint cloud of finely coagulated protein appears in two minutes. The nature of the reagent and the reliability of the test recommend this as a good, rough "bed-side" test. Whenever possible, however, the protein should be quantitated, both to check the naked-eye estimation and for record for future comparison. The following quantitative test is recommended:

(B) *The Denis-Ayer Sulphosalicylic Acid Method for Total Protein.*—Measure into a test-tube 0.6 c.c. of spinal fluid. To this add 0.4 c.c. of distilled water and 1 c.c. of a 5 per cent. solution of sulphosalicylic acid. The contents of the tube are then mixed by inversion (but not by violent shaking) and after being allowed to stand for five minutes, the suspension is read by means of a suitable colorimeter against a standard protein suspension, prepared at the same time as the unknown. This standard is made by adding to a test-tube 3 c.c. of a solution containing 0.3 mg. of protein per c.c. and 3 c.c. of 5 per cent. sulphosalicylic acid solution. The standard protein solutions are prepared from fresh normal human blood serum by the following method: Twenty cubic centimeters of serum are mixed with 200 c.c. of 15 per cent. sodium chloride solution and the resultant mixture is filtered. The nitrogen of the mixture is then determined by the Kjeldahl method, and from this strong standard, preserved with chloroform in a tightly stoppered bottle, there is prepared by suitable dilution with distilled water, two dilute standards containing respectively 0.2 and 0.3 mg. of protein per c.c. These are not reliable for a longer period than two weeks. The 0.3 mg. standard is suitable for a large majority of fluids examined; occasionally the standard is too concentrated, when a standard containing 0.2 mg. protein per c.c. may be employed. It was stated that 0.6 c.c. of fluid should be used for this determination. This will frequently be found too much in fluids with an increased protein content. With such

it is necessary to use 0.3, 0.2 or even 0.1 c.c. of fluid and to add 0.7, 0.8, or 0.9 c.c. of water, to bring the volume of the diluted fluid to 1 c.c. In fact, in fluids of extremely high protein content it is sometimes necessary to make a preliminary dilution with water, as even 0.1 c.c. of such fluids may contain too much protein to read against the standard. The colorimeter best suited to this work is the small model Duboscq with 30 mm. scale. As the cups of this instrument have a capacity of only 2 c.c., it is excellently suited for work in which only a small amount of fluid is available. The calculation of the results is simply $\frac{4}{R} \times$ 100, giving milligrams of protein per hundred cubic centimeters of fluid. R is the reading of the unknown.

It has been found that in syphilis of the nervous system the protein increase is often due principally to globulin and it is certainly true that a strong globulin reaction is usually obtained in general paresis. Therefore it is well to test for globulin separately.

(*C*) *The Ross-Jones Globulin Test.*—Underlay 0.5 c.c. of spinal fluid in a small test-tube by means of a pipette with 0.5 c.c. of saturated solution of ammonium sulphate. In ten minutes a white ring at the junction of the fluids denotes globulin. Normally no clouding occurs. A stock saturated solution of ammonium sulphate is kept with undissolved crystals in the bottle.

4. **Cell Examination.**—This includes the estimation of the number of cells as well as the kinds of cells in the spinal fluid.

Method for Estimating the Number of Cells per Cubic Millimeter.—All counts should be made as soon after obtaining the fluid as possible, for the cells degenerate rapidly. The Thoma-Levy counting chamber, Burker double type with two Neubauer rulings (making it possible to make two preparations simultaneously) is undoubtedly the most satisfactory, as it permits the use of a high dry objective, so that red blood cells may be recognized and not enumerated as white cells. A pipette made by drawing out glass tubing may be used, and the fluid examined

without being diluted.   The cover-glass is placed on the count-
ing chamber and from the pipette a drop of the spinal fluid
(previously shaken) is run under the cover-glass over each central
glass platform.   The size of the drop should be gauged so that
it is just sufficient to cover the platform and not large enough to
enter the moat.   No bubbles are permissible.   With a high
dry objective, all of the white cells in at least five large squares
(5 sq. mm.) are counted; the depth of the fluid being o.1 mm.,
the calculation of the cell count is made per cubic millimeter of
fluid.   To disintegrate any red cells and to aid in differentiating
white cells, a preparation, technique as above, may be made by
diluting and mixing the spinal fluid with an equal quantity of the
following solution, just before placing in the counting chamber:

Methyl violet,	o.1 gram;
Acetic acid,	2.0 c.c. ;
Distilled water,	50.0 "

If the spinal fluid contains blood, the leukocytes due to blood
may be allowed for by counting the red cells and substracting
one leukocyte for each 700 red cells.   In general, more than
ten cells per cubic millimeter is a pleocytosis.   But the value
of the quantitative estimation is influenced by the kind of cell,
which gives more information for diagnosis and prognosis.
    The kinds of cells are:
    1. Cells derived from the arachnoid.   These are cells with
eccentric nuclei and phagocytic properties.   They may be
classed as large mononuclears and are normal constituents of
the fluid, but are increased in inflammatory conditions.
    2. Lymphocytes, small and large.   These occur in varying
numbers, and are the predominating cells in syphilitic involve-
ment of the central nervous system, in tuberculous meningitis,
in acute poliomyelitis and in all chronic inflammatory processes.
    3. Polymorphonuclear leukocytes.   These occur in all acute
inflammatory meningeal reactions.
    Besides these three kinds of cells, commonly met with in
examinations, other cells may be found, viz. eosinophiles,
plasma cells and myelocytes.

Method for Determining the Kinds of Cells.—A practical differential count of cells can be made in the counting chamber if the spinal fluid is diluted with the methyl violet solution described above. But to make a more accurate examination of the cells, permanent stained preparations are necessary. This is not difficult with fluids containing a high cell count, as in marked meningeal reactions. But in fluids with slightly increased number of cells, as in syphilitic involvement of the central nervous system, with 20 to 60 cells per cu. mm., a special technique is needed and is as follows: One or two cubic centimeters of spinal fluid as fresh as possible and after shaking to suspend sedimental cells, are put into a small test tube; 2 or 3 drops of 20 per cent. neutral formalin are added to preserve the cells, and the mixture centrifuged at moderate speed for twenty to thirty minutes. The supernatant fluid is poured off, and the sediment transferred to a clean slide, marking the area covered with a glasscutting pencil. This is allowed to dry in the air, and is then stained with very dilute solutions of methylene blue or toluidin blue; Wright's stain gives a better differential if the cells are stained immediately after withdrawal of the fluid. The cells are from an atonic fluid, so they tend to over-stain. After staining, the preparation is allowed to dry and may be placed for an indefinite time in cedar oil for clearing. The stained, cleared smear is eventually mounted in balsam. The nuclei of the cells should stand out definitely clear.

5. **Wassermann Test.**—This test may be made as described for the blood serum (see page 539), but the quantity of spinal fluid used in the test should be as much as 1 c.c. although positive reactions may be obtained in many cases with much less.

6. **Colloidal Chemical Tests.**—In working with colloidal solutions it is of fundamental importance to keep in mind that such solutions differ materially in both physical and chemical reactions from the reactions of crystalloidal solutions; and that colloidal chemistry is a subject for research.

1. *The Colloidal Gold Test.*—(a) *Glassware.*—It is essential that the glassware has not been used for any other purpose. Erlenmeyer flasks, 2 liter, of "pyrex" glass, smaller flasks of

"pyrex," and pipettes, are needed. Each pipette and each flask for reagents must be reserved for only one solution and must not be interchanged. The pivotal point for the making of a satisfactory solution is *absolute cleanliness* of all glassware. This is obtained by first boiling in suds of ivory soap, having the water well above the glassware. Then each piece must be thoroughly rinsed with tap water several times, and then with distilled water, and finally completely filled with distilled water and stoppered until needed for use when rinsed carefully with triply distilled water. The necessity for complete cleanliness cannot be over emphasized.

(*b*) *Water.*—All water used both for reagents and for the gold-sol solution should be triply distilled.

(*c*) *Reagents.*—All chemicals must be of c. p. quality.

1. One per cent. solution of gold chloride; 1 gram to 100 c.c. of triply distilled water.

2. Two per cent. solution of potassium carbonate; 2 grams to 100 c.c. of triply distilled water.

3. One per cent. solution of formaldehyde, 40 per cent. Merck blue label; 2.5 c.c. to 100 c.c. of triply distilled water.

Technique for making 1000 c.c. of colloidal gold solution:

10 c.c. of the 1 per cent. solution of gold chloride.
 8 c.c. of the 2 per cent. solution of potassium carbonate.
 6 c.c. of the 1 per cent. solution of formaldehyde.

The gold chloride and the potassium carbonate are added to 1 liter of cold, triply-distilled water in a 2 liter flask. The contents are heated until just at the boiling point, when the formaldehyde is added drop by drop until appearance of color, when the remainder of the 6 c.c. (should be after 4.5 to 5 c.c. have been added) are added at once and the flask removed immediately, and shaken vigorously; then set aside to cool, covered.

Requirements for a satisfactory solution:

1. Must be absolutely transparent.
2. Must have a "murkiness."

3. Must react neutrally, brown-red color, to alizarin-red indicator.

4. Five cubic centimeters must be wholly precipitated by 1.7 c.c. of a 1 per cent. sodium chloride solution within one hour.

5. Must give a typical paretic curve with a known paretic cerebrospinal fluid.

6. Must give a negative reaction with a known negative cerebrospinal fluid.

Procedure of the Test.—1. Place 10 clean, dry test-tubes in a rack.

2. Put into the first tube 1.8 c.c. of 0.4 per cent. sodium chloride solution.

3. Put into each of the remaining 8 tubes 1 c.c. of the 0.4 per cent. sodium chloride solution.

4. Add to the first tube 0.2 c.c. of the cerebrospinal fluid to be tested.

5. Mix thoroughly, withdraw 1 c.c. and add to the second tube; mix again thoroughly, withdraw 1 c.c. and add to the third tube. Continue this process through the tenth tube, discarding the last 1 c.c. withdrawn.

This leaves each tube with 1 c.c. of the various dilutions, from 1:10 to 1:5120.

6. To each of these 10 tubes add 5 c.c. of the colloidal gold solution.

The color changes are read after the tubes have stood overnight at room temperature.

Reading of color changes:

a bluish tint to the original color is recorded as 1
a lilac-like color   "   "   "   2
a definite, distinct blue   "   "   "   3
an almost water-clear   "   "   "   4
complete decolorization   "   "   "   5

The readings are best considered as curves (see Fig. 148); a general paretic curve is one with the most marked changes occurring in dilutions 1:10 through 1:160, shading down to no change.

A typical general paretic curve gives, for instance, 5555543210. This curve is not limited to general paresis but is characteristic of acute, progressive, multiple sclerosis, and frequently accompanies other intense, syphilitic processes of the central nervous system.   The luetic curve, obtained frequently in tabes dorsalis and in most so-called cerebrospinal syphilis, gives typically changes as 0123332100; the most marked change occurring in dilutions 1:40 through 1:320.   The meningitic curve, which occurs inconstantly in meningitis, more frequently with the purulent cases, gives color changes more intense in dilutions 1:320 through 1:2560; e.g., 0012345554.
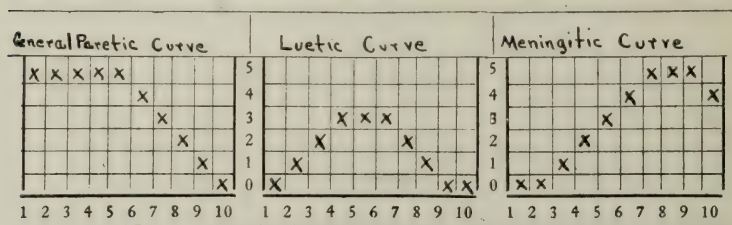
Fig. 148.

Many pathological cerebrospinal fluids give changes in the colloidal gold test.   Consequently colloidal gold reactions are of importance only as a part of the whole laboratory picture, which has in turn its fulfillment when correlated with clinical signs and symptoms.

2. *Other Colloid Tests.*—(*a*) *The Mastic Test.*—The value of the test is impaired by the influence which a slight change in the OH^- or H^+ concentration of the solutions has on the results. It may be a diagnostic aid when it is impracticable to carry out the colloidal gold test.

(*b*) *The Benzoin Test.*—The French literature reports the benzoin test as giving more constant results than the mastic. Precipitation of the colloidal suspension of benzoin occurs with fluids from syphilitic involvement of the central nervous system and in tuberculous meningitis.

Methods of Value in Research.—These laboratory tests are noted only as suggestive of possible research problems, since the procedure requires special laboratory facilities and trained technique.

1. The determination of non-protein nitrogen.[1]
2. The determination of urea.[1]
3. The determination of uric acid.[1]
4. The determination of chlorides, Whitehorn Method.[1]

The methods used on cerebrospinal fluid are identical with those used for blood analysis. The standards, however, must be diluted to compare with the smaller amounts of the constituents in the spinal fluids.

7. **The Quantitative Estimation of Sugar.**—Qualitative tests for sugar in spinal fluid are of slight value, for such tests do not prove an increase or a decrease of the normal amount, on which depends diagnostic significance. An increase in the sugar may occur in many pathological conditions, such as tumors, acute poisoning, acute infections, etc., without being of differential diagnostic importance; whereas the consensus of opinion is that in acute purulent meningitis there is a complete absence of sugar in the fluid. But quantitative determinations are of value in differentiating tuberculous meningitis, in which the amount of sugar is markedly reduced, from encephalitis lethargica, in which the amount of sugar is increased.

Folin and Wu Method.—Special solutions used are:

1. *Standard Sugar Solution.*—Dissolve 1 gram of pure anhydrous dextrose in distilled water and dilute to a volume of 100 c.c. Mix, add much toluene, and bottle. From this take 1 c.c. and dilute to 200 c.c. for 0.005 per cent. solution.

2. *Alkaline Copper Solution.*—Dissolve 40 grams of anhydrous sodium carbonate in about 400 c.c. of distilled water and

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[1] Folin, "Laboratory Manual," 1922 edition. Appleton. (*a*) V. Kafka, *Deutsche med. Wchnschr.*, 47, 1422, Nov. 24, 1921. (*b*) E. Sahlgren, *München. med. Wchnschr.*, 69: 618–19, April 28, 1922. G. Guillain, Guy-Laroche, and P. Lachelle, "Réaction de Précipitation du Benjoin."

Colloidal avec les Liquides cephalozrachidiens Pathologiques. *Compt. Rend. de la Soc. de Biol.*, 1077, July 17, 1920.

Ibid., 1199, July 31, 1920.

transfer to a 1 liter flask. Add 7.5 grams of tartaric acid and when the latter has dissolved, add 4.5 grams of crystallized copper sulphate; mix, and make up to a volume of 1 liter. If the carbonate used is impure, a sediment may be formed in the course of a week. If so decant the clear solution into another bottle.

3. Molybdic-phosphoric reagent: Transfer to a 2 liter beaker or large flask 35 grams of molybdic acid and add 5 grams of sodium tungstate. To this add 200 c.c. of 10 per cent. sodium hydrate and 200 c.c. of distilled water. Boil for twenty to forty minutes or longer to drive off the ammonia (molybdic acid some times contains large amounts of ammonia as impurity). Replace the water evaporated if necessary. Cool, then dilute to 350 c.c. and add 125 c.c. of phosphoric acid, 85 per cent., and dilute the whole to 500 c.c.

The Test: To 2 c.c. of cerebrospinal fluid, measured with a Folin-Ostwald pipette, 14 c.c. of distilled water are added; to this is then added 2 c.c. of a 10 per cent. solution of sodium tungstate and 2 c.c. of a $\frac{2}{3}$ normal solution of sulphuric acid.

Mix by rotation; then allow to stand for fifteen minutes. Filter through three filter papers.

Put 2 c.c. of filtrate into a Folin blood sugar tube, graduated at 25 c.c.

Add 2 c.c. of the alkaline copper solution.

Place the tube in boiling water for six minutes. Then place in a beaker of cold water, and immediately add 2 c.c. of the molybdic-phosphoric reagent.

An intense blue color develops, formed by the reaction of the molybdic-phosphoric reagent with the cuprous oxide, which is reduced from cupric sulphate by the sugar in the spinal fluid. Simultaneously with the above, there is run a standard determination for comparison, using 2 c.c. of the 0.005 per cent. dextrose solution, made from the stock solution described above.

Both tubes are diluted to the 25 c.c. mark and a colorimetric comparison made in a 60 mm. Duboscq colorimeter. But before attempting to determine the color of the unknown, read the standard against itself at 20 mm. until the correct value is

obtained (the error should not exceed 0.2 mm.). After rinsing the right hand cup and plunger with the unknown then fill the cup to same depth as standard and determine the unknown's color value in terms of the known standard, taking the average of at least six readings. Calculation: $\dfrac{20}{R} \times 0.05 \times 10 \times 100$, which may be simplified by using the formula: $\dfrac{1}{R}$. R is the reading of the unknown.

8. **Bacteriological Examination.**—In meningitis the spinal fluid is more or less cloudy from cellular exudation, but in tuberculous meningitis this may be so slight that the fluid appears clear unless examined carefully.

The fluid should be examined by both cultures and smear preparations.

In making cultures, to avoid contamination, and also to prevent the dying out of the labile meningococcus, it is advantageous to allow the fluid to flow directly from the puncture needle into the culture tube. In some cases, however, it may be better to make the cultures from the sediment after centrifuging in a *sterile* tube.

For growing Bacillus influenzæ blood agar is necessary; for the meningococcus, blood agar, hydrocele agar, or "hormone" agar, are more favorable than ordinary media.

Smear preparations may be made from the fluid directly or from the centrifuged sediment and may be stained in various ways. For demonstrating the tubercle bacillus two methods are helpful:

1. *Clot Method.*—Most fluids from tuberculous meningitis show a fine web-like clot on standing, provided that the fluid has not been agitated too much. This clot may be removed with a wire and placed on a slide, or floated out onto a slide in a Petri dish. It is spread out, dried, fixed, and stained for tubercle bacilli.

2. *Coagulation Method.*—The protein in the spinal fluid is coagulated by mixing the fluid with 3 parts of alcohol. The suspension of coagulated protein is centrifuged at high

speed, the sediment taken up with a fine capillary pipette, and placed on a slide or cover-glass, where it is allowed to dry. It is then fixed and stained for tubercle bacilli.

In both methods care should be observed to keep the material to be stained on a small area of the slide or cover-glass.

The tubercle bacillus may also be demonstrated by the guinea-pig inoculation test, which is best carried out by subcutaneous or intraperitoneal injection of the centrifuged sediment of the fluid, obtained in a *sterile* tube.

EXAMINATION OF THE SPUTUM

The secretion raised from the air-passages by coughing is almost invariably contaminated with the secretion of the naso-pharynx and with particles of food from the mouth. In exam-inations of sputum these contaminations must always be borne in mind. The amount raised varies from a few cubic centi-meters to one or even several liters in twenty-four hours.

The macroscopic appearances of the sputum depend on the varying proportions of mucus, pus, blood, and serum present. The tenacity is mainly due to the mucus. The reaction is usually alkaline.

The general color, consistence, and separation into layers is best seen after the sputum has stood for some time in a tall glass. For more careful macroscopic examination small portions of the sputum are transferred to flat glass dishes, where they are spread out thinly by needles and examined over black or white paper. Porcelain plates painted black or black paper itself can be used. The latter method is con-venient, because the sputum can be burned up with the paper.

The constituents of the sputum which may be recognized macroscopically are few in number, and not so important as those which may be found microscopically.

Macroscopic Examination.—1. *Caseous Masses.*—In the sputum from tubercular cases small, opaque, yellowish-white masses from the size of a pin-head to that of a small pea can occasionally be found, which spread out beneath a cover-

glass like a bit of cheese. They are small caseous masses which are valuable for microscopic examination because they usually contain tubercle bacilli and elastic fibers.

2. *Fibrinous casts of the bronchioles* can usually be found in the sputum from the third to the seventh day in cases of acute lobar pneumonia. They appear as yellowish-white or reddish-yellow threads, 2 to 3 mm. thick and $\frac{1}{2}$ to several cm. long, and are often branched. The large ones are often rolled into balls, and show best after being shaken in water. Casts of the bronchi are found in cases of fibrinous bronchitis.
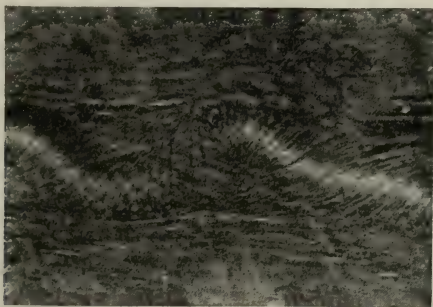
FIG. 149.—Curschmann's spiral; × 425 (W. H. Smith; photo. by L. S. Brown).

3. *Curschmann's spirals* (Fig. 149) of twisted threads of mucus enclosing epithelial cells and leucocytes occur rarely, except in bronchial asthma. They appear macroscopically as grayish-white or whitish-yellow masses or threads, $\frac{1}{2}$ to $1\frac{1}{2}$ mm. thick and $\frac{1}{2}$ to 8 cm. long, and often show a visible spiral arrangement.

4. *Dittrich's Plugs.*—These are whitish-yellow masses from the size of a pin-head to that of a bean, which are formed in cases of putrid bronchitis and of gangrene of the lung. They have a very fetid odor, a cheesy consistency, and are rather easily compressed. Besides organisms they contain numerous fat-crystals.

5. *Shreds of tissue* are found almost solely in gangrene of the lung, and are best recognized with the microscope.

6. *Concretions*, portions of cysticercus membrane, etc., are rare in the secretion from the lungs.

Microscopic Examination.—Microscopically, the sputum may show various kinds of cells, fragments of tissue, including elastic fibers, vegetable and animal parasites, and crystals.

They will be taken up in order:

1. *Red Blood-globules.*—In fresh hemorrhages they appear normal, often in rouleaux. In old sputa many have lost their color.

2. *White blood-corpuscles* are almost invariably polynuclear, and the majority of them contain neutrophilic granules. In asthma, however, numerous eosinophilic and rather numerous basophilic leucocytes are regularly found. The leucocytes often contain pigment- or fat-granules. The pigmented cells found almost wholly in chronic passive congestion of the lungs are endothelial leucocytes. The pigment appears as yellowish, yellowish-red, or brownish-red granules or as yellow diffuse pigmentation. Occasionally, however, it surrounds granules of carbon, and then appears brownish or grayish-black. The pigment is derived from the blood, and will usually give the iron reaction, but very young or old pigment will not.

3. *Epithelial Cells.*—Pavement, cylindrical, and ciliated cells are found. The first come from the naso-pharynx; the others usually from the trachea and bronchi, but may come from the nose. Desquamated alveolar epithelium is difficult to demonstrate.

4. *Fatty Detritus.*—Fat-drops are frequently found, due to the fatty degeneration of cells.

5. *Elastic fibers* (Fig. 147) occur singly, but more often as a network. They are recognized by their sharp, dark outlines due to their high degree of refractiveness, and by their marked degree of resistance to acids and alkalies by which other like tissues, such as connective-tissue fibers, are destroyed. Elastic fibers are most abundant in the caseous masses above mentioned. When these masses cannot be found, the thicker portions of the sputum are squeezed between a slide and cover-glass, or between two slides, and examined with a low power. The examination is rendered easier by mixing a little sputum with a 10 per cent. solution of caustic potash or soda. In

certain cases it is necessary to mix together equal parts of the sputum and 10 per cent. caustic potash or soda, and to boil the mixture until the sputum is dissolved. The solution is then mixed with four times its own volume of water and allowed to stand for twenty-four hours, when the sediment can be examined for the elastic fibers

Vegetable and Animal Parasites.—Of the vegetable parasites, the most important is the tubercle bacillus. Other bacteria sometimes examined for are the pneumococcus, the influenza bacillus, and the actinomyces.
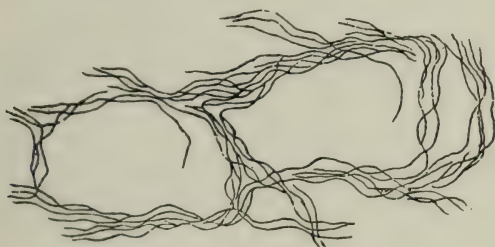
FIG. 150.—Elastic fibers (after Strümpell).

W. H. Smith's Method of Staining Bacteria in Sputum.— This has been found particularly useful in demonstrating the pneumococcus in the sputum. The sputum or other material should be fresh. The cover-glasses should be spread as thinly as possible and fixed by passing three times through the flame in the usual manner.

1. Stain in aniline-gentian-violet solution for a few seconds, gently warming until the staining fluid steams.

2. Wash in water.

3. Cover with Gram's solution of iodin for thirty seconds.

4. Wash with 95 per cent. alcohol until the color ceases to come out.

5. Wash with ether for a few seconds. (To remove fat.)

6. Wash in absolute alcohol for a few seconds.

7. Stain one to two minutes in a saturated aqueous solution of eosin.

8. Wash with absolute alcohol for a few seconds.

9. Clear with xylol.

10. Mount in balsam.

The pneumococcus is stained blue-black, while the capsule is stained pink. This method gives beautiful preparations. With the following modification it has been used by Smith as a routine stain for sputum. The advantage of this modification is that influenza bacilli and other bacteria which do not stain by Gram's method are clearly brought out, as are also
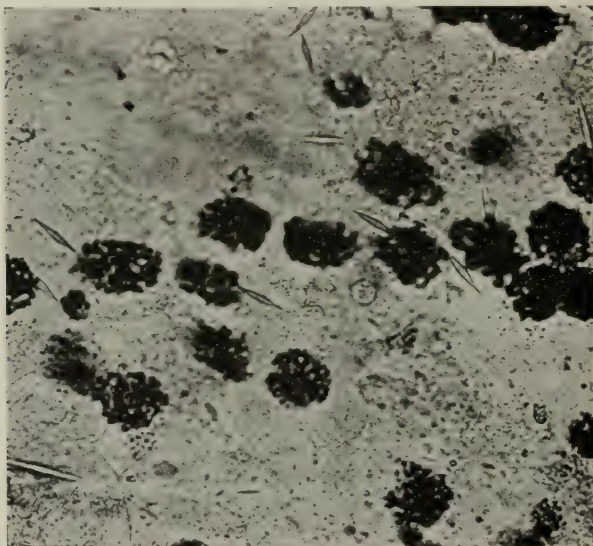
Fig. 151.—Charcot-Leyden crystals and eosinophilic leukocytes in a smear preparation of sputum from a case of asthma (W. H. Smith; photo. by L. S. Brown).

eosinophilic leucocytes. This modification consists in washing the preparation with Löffler's alkaline methylene-blue solution just after it has been stained with eosin, as described above, and then, after the excess of eosin has been removed by the methylene-blue, steaming the methylene-blue solution for a few seconds while on the cover-glass. The preparation is then washed in water, rinsed with alcohol, cleared with xylol, and mounted in balsam.

Of the animal parasites, the entamœba histolytica is sometimes found secondary to an hepatic abscess which has perfo-

rated into the lung. Portions of the membrane of an echino-coccus cyst or the hooklets from the head may be found in the sputum, but infection with this parasite is very rare in this country.

Of the crystals which occur in sputa, the most important are the Charcot-Leyden crystals, found mainly in bronchial asthma, and the crystals of the fatty acids, of cholesterin, and of hematoidin. Tyrosin and leucin are much more rare.
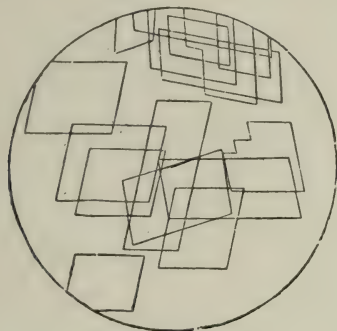
FIG. 152.—Crystals of cholesterin (after Strümpell).

The **Charcot-Leyden crystals** are colorless, elongated octahedra of varying size, soluble with difficulty in cold water, insoluble in alcohol, ether, chloroform, and dilute saline solution.

Hematoidin crystals occur as ruby-red rhombic plates or columns.

Cholesterin crystals (Fig. 152) occur as the well-known small and large rhombic plates.

The **fatty-acid** crystals occur as long, pointed needles, either singly or in groups. They are easily soluble in ether or hot alcohol, insoluble in water and acids.

POST-MORTEM EXAMINATIONS

Introduction.—The method of making post-mortem examinations most generally followed in this country and abroad is that originally taught by Virchow. It has been variously modified in minor details by his pupils and followers. The strongest adverse criticism which can be made of the method is that it works best when the various organs in the body are nearest normal. Its chief fault lies in the early separation of the different structures from each other, so that interesting pathologic relations are often overlooked and destroyed. Special procedures are advised for certain conditions, but emphasis is not placed on them.

Two other methods of making post-mortem examinations were developed besides that of Virchow's, but have never attained the same general recognition, although both contain principles of great value.

The technique of Rokitansky, lately best exemplified by Chiari, appeared in printed form a number of years ago. Its fundamental principle is to examine and open every organ *in situ* before removing it, so that all abnormal relations between organs, blood-vessels, and other structures can be discovered and preserved intact.

Recently, Hauser has published Zenker's post-mortem technique. Its main principle is to remove the organs in block, so that they can be examined from every side and incised before they are separated from each other.

In both methods the incisions in the various organs, and especially the heart, differ more or less radically from those recommended by Virchow.

A knowledge of all three forms of procedure is useful. It broadens one's point of view, and tends to keep one's mind open to possibilities. A list of the best publications on post-

mortem technique is appended at the end of this section of the book.

The problem offered by an autopsy is often solved in part or wholly by the macroscopic post-mortem examination. More frequently, however, the complete and final solution is reached only after careful bacteriological and histological study. The post-mortem examination may, therefore, be looked upon as the beginning of the solution of the problem. Its particular function is to demonstrate in the individual case all congenital or acquired abnormalities, all macroscopic lesions, and to explain all gross mechanical questions. It furnishes the material for bacteriological and histological study. Perfectly to accomplish its purpose a post-mortem examination must be made in a careful, systematic manner.

While a general method of procedure is advisable, it will often be found advantageous, or even necessary, to depart from it. According to Orth, "the chief requisite of every exact post-mortem examination is this, that no part shall be displaced from its position until its relations to the surrounding parts are established, and that no part shall be taken out by whose removal the further examination of other parts is affected."

The order and method of procedure in making a post-mortem examination, including the various incisions, may be said to have been planned for the routine examination of normal or diffusely diseased organs. As soon as a noticeable focal lesion is present the order of procedure and the customary method of removal and of incision must be so altered as best to display the lesion.

Instruments.—The following instruments will be found extremely useful in the autopsy-room, although not all of them are necessary:

The *autopsy-table* should be large, so as to accommodate on it the instruments and several dishes in addition to the body. It should have a slightly raised edge, and should slope gently toward an opening in the center for the escape of fluids. The table is best made of zinc, and along one edge should have a

centimeter scale. The water for use on the table is best supplied by a rubber tube from an overhead pipe reaching to within 60 to 100 cm. of the table.
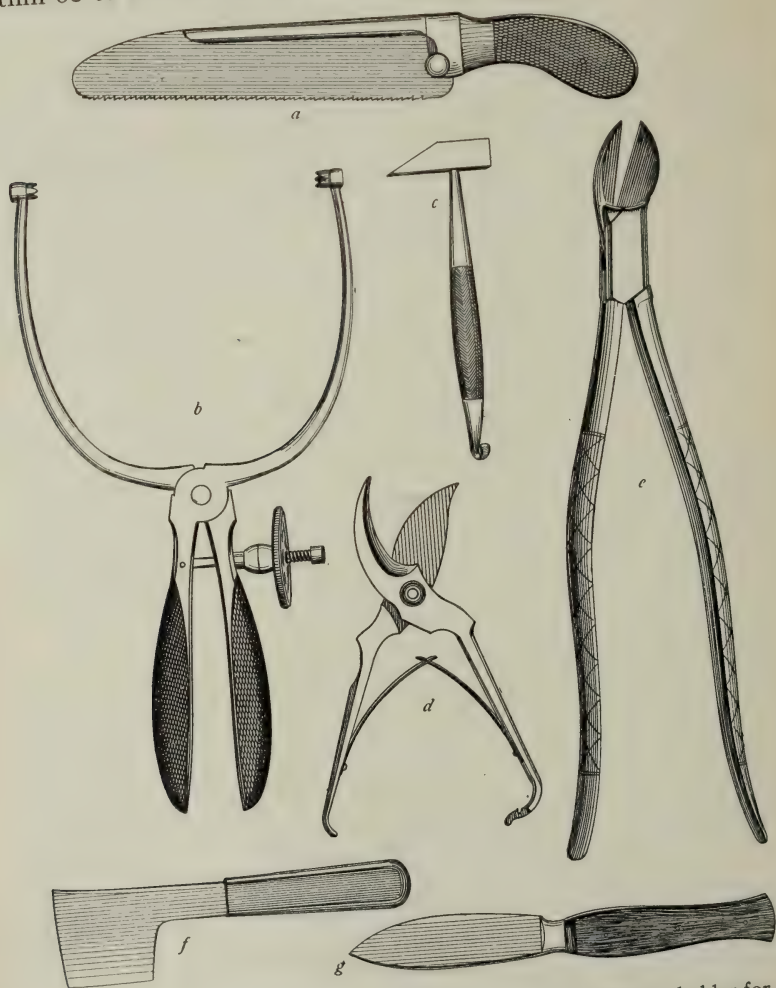
FIG. 153.—Instruments for use in the autopsy-room; *a*, Saw; *b*, holder for the head; *c*, steel hammer with wedge end and blunt hook on the handle; *d*, costotome; *e*, bone-cutter; *f*, hatchet-chisel; *g*, autopsy-knife.

The *scales* for weighing the various organs should have a large pan and gram and kilogram weights.

A *band-saw* will be found very useful for sawing bones for the inspection of the marrow, and for calcified and osseous tumors.

The best *autopsy-knife* is a stout, broad-bladed knife with bellied edge and heavy handle. The blade should measure about 12 cm. in length and 3 cm. in width; the handle should
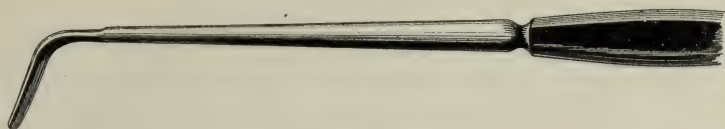
FIG. 154.—Myelotome.

be 12 cm. in length. Many operators prefer a somewhat smaller knife than this.

Amputating-knives of different sizes are useful for long, deep cuts into organs and tumors.

A *myelotome* is a short, thin, narrow knife-blade, 1.4 cm. long and 4 mm. wide, set obliquely on a slender steel stalk ending in a wooden handle (Fig. 154). It is used only for cutting the cord squarely across in removing the brain.
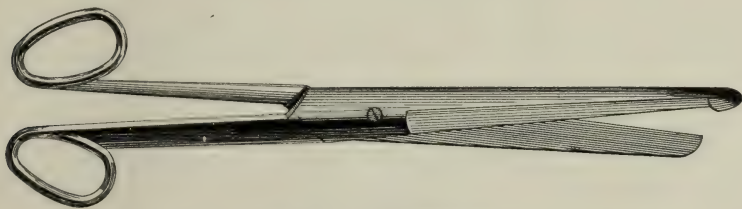
FIG. 155.—Enterotome.

Cartilage-knives and *scalpels* of different sizes are useful for a variety of purposes.

Scissors, both straight and curved, should be of various sizes. A medium-sized and a fine pair should each have one probe-pointed blade.

An *enterotome* is a long, straight pair of scissors, of which one blade is longer than the other and blunt at the extremity (Fig. 155). A hook at the end is not advisable. The instrument is used in opening the intestines and the heart.

A *saw* with movable back and rounded end will be found the most generally useful for opening the skull and the spinal canal.   An ordinary meat-saw is preferred by some, but cannot be used on the vertebræ.

Luer's double rachiotome, or adjustable double saw (Fig. 156), is very useful in removing the cord, and is the safest instrument to put into the hands of beginners.

Forceps: several sizes, large and small, mouse-toothed.

Costotome: heavy bone-shears for cutting the ribs.
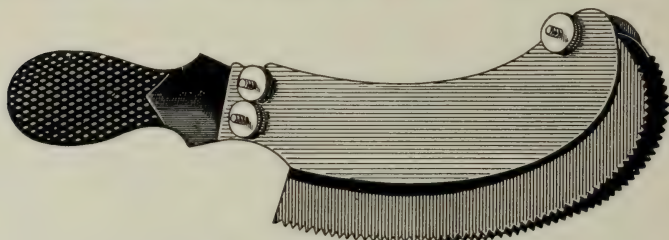
FIG. 156.—Luer's double rachiotome.

A powerful *bone-cutter*, with short blades, 5 cm. long, set at an angle of about 45° to the handles, which are 36 cm. in length, is employed for dividing the arches of the cervical vertebræ and for other purposes where ordinary bone-cutters will not do.

A *chisel* with 2 cm. cutting edge, for exposing the marrow of the long bones, removing portions of the base of the skull, etc.

A *hatchet-chisel* of steel for starting the calvarium and spinous processes after sawing the skull and the vertebral column.

Soft-iron hammer with wooden handle.

Steel hammer with wedge end, and blunt hook on the handle.

Holder for the head while sawing the skull.

Autopsy-needles, long and a little curved.

Probes of flexible metal; also fine glass probes for small blood-vessels or ducts.

Grooved director.

Pans for holding water, organs, etc.

Boards, square or oblong, 30 × 30 or 30 × 50 cm., on which to lay instruments or to cut organs.

Sponges.

Catheters.

Strong *hemp twine* is the best for sewing up the body.

Glass graduates for measuring fluids.

A *block of wood* with shallow depression for the neck; for use while opening the head.

Vise.

Small *cup* or *dish* for removing fluid from cavities.

General Rules.—The room for an autopsy should be well lighted, otherwise the finer changes in the tissues cannot be recognized. Artificial illumination is not good, because the colors of the tissues are entirely changed by the yellowness of the light.

Before beginning an autopsy the necessary instruments should be arranged on a short board on the autopsy-table in the order in which they are most likely to be used.

The operator stands on the right side of the body. This position he rarely leaves except for some definite purpose; for example, in opening the skull he stands at the head.

Order and cleanliness are the first points to be insisted upon at every autopsy. Clean water should always be at hand for washing the instruments and for keeping the hands free from blood and pus. The cut surface of an organ should not be washed with water except to remove blood; gently scrape the surface with the knife held obliquely.

In cutting, the knife should be drawn, not pressed or shoved into the tissues. According to Virchow, a broad, clean cut into an organ, even if incorrectly made, is much better than several short cuts which leave a ragged surface.

The autopsy-knife should be grasped in the hand as if to cut bread. In using this knife the main movement should be from the shoulder, not from the wrist as in dissecting. It goes without saying that the sharper the knife the better.

In cutting the brain and cord, especially if their consistence is lessened, moisten the knife to prevent the tissue from sticking and tearing.

Before beginning an autopsy it is important to know the main points in the clinical history of the case, as they may greatly lighten the work of investigation by calling attention to those organs that require special examination.

The record of an autopsy should be dictated by the operator as he proceeds with the examination of the body, and should be as nearly as possible an objective description of the appearances found. Only the anatomical diagnosis should express the opinion of the operator. If it is not convenient to dictate the autopsy during its performance, the description of the lesions certainly ought to be made with the organs in sight, and not from memory after the lapse of hours or even days, when many of the details may be forgotten. Later, the results of the bacteriological and histological examinations should be added to the autopsy report, so as to make the record complete.

The thin rubber gloves now used by surgeons are very useful in making post-mortem examinations, especially in septic cases and while opening the stomach and intestines. Rubber cots for the fingers are often useful.

For cuts on the fingers use celloidin dissolved in equal parts of alcohol and ether, instead of flexible collodion, because the latter will not stick. A cut received during an autopsy should immediately be washed thoroughly. For protection during the rest of the autopsy, use a rubber glove or cover the cut with celloidin.

After an autopsy the operator should scrub his hands thoroughly with soap and brush, just as a surgeon does before an operation, and then use, if he so desires, an antiseptic solution, such as corrosive sublimate (1 : 2000) or 70 per cent. alcohol. For removing odors from the hands, turpentine will often be found serviceable, or a saturated solution of permanganate of potassium followed by oxalic acid.

For infections of slight wounds, such as scratches, or such as occur in hair-follicles, the best treatment within the first twenty-four hours is to bore into them with a sharp-pointed orange-wood stick dipped in strong carbolic acid, followed by washing with 95 per cent. alcohol. The procedure is prac-

tically painless, and the infection is stopped in the very beginning. Where the infection has spread, surgical treatment must be restored to.

Suggestions to Beginners.—In a case of *general miliary tuberculosis* the older focus from which the organisms have spread must always be found. Look especially for tubercular thrombi in the pulmonary veins as a frequent source of the general infection.

In a case of *embolism* hunt for the thrombus, bearing in mind, however, that the whole of a thrombus may become free and form an embolus. An arterial embolus may be due to a venous thrombus, in which case it must have passed through an open foramen ovale, except in the case of thrombi of the pulmonary veins.

In *acute peritonitis* always seek for a source of infection (appendix, female genitals, gastro-intestinal tract, etc.). It cannot always be found.

In *hemorrhage from the stomach* associated with cirrhosis of the liver look for rupture of dilated esophageal veins.

In cases of more or less *sudden death*, especially if preceded by signs of asphyxia, always examine the pulmonary artery *in situ* for possible emboli. In cases of *instantaneous death* examine the coronary arteries.

Private autopsies must often be made under many disadvantages, and, when out of town, not infrequently in a short space of time. It is always important to warn the attending physician not to allow the undertaker to inject the body before the autopsy, because the color and consistence of the organs are so changed by most injecting fluids that it is difficult to recognize the pathological processes. If there is danger of post-mortem changes, have the body packed in ice.

A regular autopsy-bag will be found very convenient for carrying to private autopsies. It is made of leather lined with rubber, and measures about 40 × 18 × 20 cm. Loose within it is carried a rubber bag 40 × 24 × 20 cm., shaped like a short envelope with a flap (22 cm. long) on one side, for bringing away any organs that demand further examination.

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The case of instruments should contain one or two autopsy-knives, two scalpels, a pair of forceps, one or two pairs of scissors, an enterotome, a steel hammer with wedge-end and with a blunt hook on the handle, a small chisel, a saw with detachable handle and back, an autopsy-needle, and a probe; free within the bag should be carried a spool of strong twine, a costotome, a long slender knife for use in removing the brain, a hammer with soft iron head, and a sponge. In rare cases additional instruments may be required. A white duck apron for personal use will always be found convenient. It is also well to carry along several blood-serum tubes and a platinum needle for making cultures at the autopsy. When there is a lesion of the nervous system it is advisable to bring a jar of a 4 per cent. solution of formaldehyde and to place the tissue in the fluid at the autopsy, as otherwise it is not easily gotten to the laboratory in good condition.

At the house can always be obtained a slop-pail, a wash-bowl, a pitcher of water, several newspapers, and an old sheet. The body is usually on an undertaker's frame, but it may be in an ice-box or on the bed. The examination of the chest and abdomen can be made in any of these positions. If, however, the body is in an ice-box, it must be raised to the level of the top of the box in case it is necessary to open the head.

The clothing on the body can be removed, or, if only a shirt or a night-dress, is best slit down the middle and turned out over the arms. Tear the sheet into four equal pieces. Fold and tuck in one piece on each side of the trunk and neck, allowing the outer portion to fall over the arms. Fold and lay the third piece on the lower extremities, tucking the upper end beneath the clothing below the pubes. The fourth piece can be placed beneath the head if it is to be opened. This procedure leaves the front of the thorax and abdomen free for operation and protects the rest of the body and the clothing. On the thighs place one or two folded newspapers, and on these the necessary instruments. On the legs place the bowl containing only a dampened sponge. If the undertaker has not put a rubber sheet on the floor beneath the body and on the side

where the operator is to stand, newspapers should be spread to protect the carpet. Place the slop-pail on the rubber sheet within convenient reach. Having thus made all arrangements, even to the threading of his needle, the operator is ready to begin.

If the cord and brain have to be examined as well as the body, it is best to do the cord first, so as to avoid the leakage that might otherwise occur from the trunk-cavities if they had been opened first. To support the head while opening it, use a stick of wood, a brick, or, in case of necessity, the instrument-box wrapped in a newspaper.

At a private autopsy cleanliness is extremely important. If there is no undertaker or nurse present, the operator himself must see that everything is cleaned and put in order before leaving, that all the blood-stains are removed from the dishes, and that all papers and soiled cloths are burned or rolled up and left in a neat bundle for the undertaker to dispose of. Ground coffee thrown on a shovelful of burning coals will be found helpful in disguising the odor in the room after an autopsy.

EXTERNAL EXAMINATION OF THE BODY

External examination is often of great importance, especially in medico-legal autopsies, and should never be neglected, as it may throw great light on lesions found within the body. It should be systematic and careful, and is best taken up in the following order:

I. **Inspection of the Body as a Whole.**

1. Sex.

2. Age.

3. The *body-length* should be measured on the table beside the body, between points opposite the vertex of the head and the sole of the foot beneath the ankle.

4. The *development of the skeleton* has reference to the bony framework, which may be powerful, slender, or deformed.

5. The *general nutrition* is shown by the amount of muscular development and of subcutaneous fat-tissue. The latter is judged by pinching up folds of skin.

6. The *general condition of the skin* includes amount of elasticity, bronzing, jaundice, edema, and decubitus.

7. *Post-mortem discolorations* may be divided into three varieties:

(*a*) *Hypostasis of blood*, or the settling of blood into the lowest lying blood-vessels; this form of discoloration disappears on pressure.

(*b*) *Diffusion of blood-coloring matter* out of the vessels into the surrounding tissues (due to blood-pigment being set free by post-mortem decomposition); does not disappear on pressure.

(*c*) The *greenish discoloration*, usually seen earliest over the abdomen, is due to sulphide of iron formed through decomposition of the tissues. This discoloration is important, as it may modify the interpretation of appearances observed in the internal organs.

8. *Post-mortem rigidity*, degree and extent. It begins in the maxillary muscles, and spreads gradually from above downward, disappearing later in the same order. It is most marked, and lasts longest, in muscular individuals who have been ill but a short time. Cholera furnishes the most marked cases. The rigor disappears quickest in cachetic diseases. When once it has been forcibly overcome, it does not recur. The time of beginning after death varies widely—from ten minutes to seven hours.

II. Special Inspection of the Different Parts of the Body.

The examination should begin with the head. Any lesion or abnormality found should be carefully noted. Particular attention should be paid to the condition of the pupils and to the color of the sclera. Then follow in order the neck, the thorax (size and shape), the abdomen (distended or retracted), the genitals, and the extremities.

INTERNAL EXAMINATION OF THE BODY

The opening of the body-cavities is described first, because the brain is relatively much less frequently the seat of disease, and because in this country it is often impossible to obtain

permission to open the head. Moreover, the lesions in the body often throw much light on those to be expected in the brain. The advantage of examining the brain first, particularly in those cases in which the important lesions are cerebral, is said to be that the amount of blood in the cerebral vessels can be more accurately determined. After the heart has been removed some of the blood in the brain may escape through the severed vessels below.

In routine examinations, however, the body is usually examined first, then the brain, and finally the cord. It is not a bad practice to remove the calvarium, to examine the meninges over the upper surface of the cerebrum, and then to make the examination of the body before removing the brain. In this way any change in the blood-supply of the cerebral vessels would be observed.

Opening of the Abdominal Cavity.—In the examination of the body the peritoneal cavity is opened first, the two pleural cavities next, and the pericardial cavity last. The cavities and their contents are to be inspected in the order and at the time that each is opened, but the organs are to be removed from the cavities for further examination in the reverse order, beginning with the heart.

The *primary or long anterior incision to bare the thorax and to open the abdomen* (Fig. 157) should extend from the larynx to the pubes, passing to the left of the umbilicus, so as not to cut the round ligament. In cutting, the handle of the knife is depressed so as to use the belly of the blade rather than the point. An incision beginning as high as the chin is, unfortunately, rarely allowable. Over the sternum the cut should extend down to the bone; over the abdomen, however, only into the muscles, or in fat people through the muscles into the subperitoneal fat-tissue. To open the abdominal cavity, nick carefully through the peritoneum just below the sternum, introduce the first and second fingers of the left hand, and while making strong upward and outward traction on the right abdominal flap extend the incision to the pubes. Some operators prefer to separate the fingers and to cut between them.

The abdominal flaps are rendered much less tense by cutting the pyramidales and recti muscles transversely from below just above the pubis.   Care must be taken not to injure the overlying skin. The abdominal cavity can now be examined, but more room will be obtained if the skin and the underlying muscles be first stripped back from the thorax to about 5 cm. outside of the costochondral line.
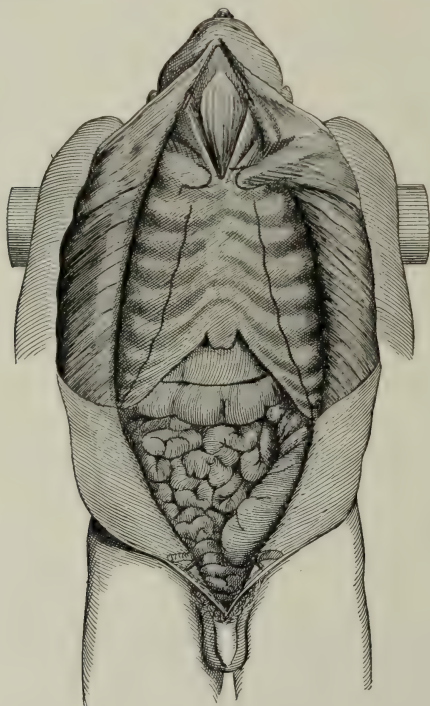
Fig. 157.—Primary incision in the body (Nauwerck).

The operation is most easily and neatly done by lifting the skin directly away from the chest-wall or turning it forcibly out with the left hand, and then cutting the tense tissue close to the cartilages and ribs with long sweeps of the knife held almost flat.   The operation begins over the lower border of the ribs and extends upward.   In dissecting off the skin and muscles from the left side the right hand works underneath

the left. The mammæ can easily be incised from the under side of the flap, and if necessary the axillary lymph-nodes can be reached by dissecting the skin farther out, especially over the clavicle. Before beginning the inspection of the peritoneal cavity it is important to examine first the surface of the incision into the abdomen, noting the thickness and color of the fat-tissue and the condition of the muscles.

Inspection of the Abdominal Cavity.—The character of any fluid present should be determined and its amount measured or estimated. The simplest way to remove it is to dip it up with a small cup or dish and pour it into a glass graduate for inspection and measurement. If the presence of gas within the peritoneal cavity is suspected, a small pouch should be formed in the first incision as soon as it has been made and water poured in. The first opening into the abdominal cavity should then be made with the point of a scalpel at the bottom of the water, through which the gas, if present, will escape in bubbles.

The various abdominal organs and their relations to each other are to be investigated *in situ* by sight and by touch. As a rule, examine first the gastro-intestinal tract, including the appendix and the mesenteric lymph-nodes. Ulcerations of the intestine can often readily be made out through the walls. The examination of the spleen, liver, kidneys, and pelvic organs follows. The pancreas is easily reached by tearing through the omentum between the stomach and the colon, so as to open the lesser peritoneal cavity.

After the inspection of the abdominal organs the position of the diaphragm is to be ascertained on both sides in the costochondral line by measuring with the right hand passed palm upward underneath the ribs, and the left hand outside at the corresponding height to mark the position of ribs or intercostal spaces. On the right side the hand is to be passed up on the outside of the falciform ligament. Normally, the diaphragm stands at the fifth rib on the left side, and at the fourth rib or fourth interspace on the right.

Opening of the Thorax.—To open the thorax, cut through the cartilages close to the ribs from the second down (about 5 mm. distant) with a scalpel held nearly horizontal, so that as one cartilage is cut through the handle of the scalpel will strike the next below and prevent the blade from penetrating too far and injuring the lung. In young people the cartilages can be cut easily by one long stroke on each side, but care must be taken not to go too deep. If the intercostal muscles are not divided by the same operation, the sternum can be depressed by the left hand and the muscles severed by one pass of the knife on each side. The lower end of the sternum can now be elevated and freed from below upward from the diaphragm and pericardium until the first rib is reached. The cartilage of this rib is to be cut about 1 cm. farther out than the others, and from below upward toward the clavicle, with the handle of the knife beneath the elevated sternum and with the point and edge of the knife directed upward and a little outward. The sternum is then to be still further freed from the anterior mediastinal tissue until its upper end is reached. The sterno-clavicular joint on the left side can now be easily opened from below by entering a scalpel just above the cartilage of the first rib, and following the irregular line of the joint around the end of the clavicle, while at the same time drawing the sternum over to the right side of the body. The right sterno-clavicular articulation is to be opened by continuing the incision of the scalpel over the upper end of the sternum and into the second joint. The advantage of this method is that there is much less danger of wounding the large vessels at the base of the neck, and thus of mingling blood with any exudation which may happen to be present in the pleural cavities. If preferred, however, the articulations can be opened and the cartilages of the first ribs cut from above before freeing the sternum from the diaphragm. In this case enter a short, sharp, narrow-bladed scalpel held vertically, but loosely, into the left joint on its upper side, starting the incision just outside of the attachment of the sternal end of the sterno-mastoid muscle, and cut around the end of the clavicle by a series of short up-and-down strokes, allowing

the blade to follow the irregular line of the joint.   After cutting through the joint continue the incision outward and cut through the cartilage of the first rib.

If the cartilages are calcified, use the costotome and cut through the ribs, as more room can be gained in this way, and they are more easily cut than calcified cartilages.   When for any reason it is not permitted to open the thorax, the organs within it can be obtained through the opening into the abdominal cavity by freeing the diaphragm from the ribs, and removing first the heart and then the lungs.   The sternum should be inspected at the time of its removal.   It is perhaps best to examine next, especially in children, the epiphyses of the ribs at the costochondral line for any evidence of thickening.

Inspection of the Pleural Cavities.—In the pleural cavities, as in the peritoneal cavity, the character and amount of any abnormal contents must be determined.   If, from the clinical history or from any other reason, the presence of air in a pleural cavity is suspected, a pouch should be formed over the ribs by aid of the skin-flap and filled with water.   The pleural cavity is then to be pierced with a scalpel through the bottom of the pouch.   Air, if present, will bubble up through the water.

Slight adhesions are best torn through or cut.   If the lungs are firmly attached, it is best to strip off the costal layer of the pleura with the lung.   This is most easily done by starting the anterior edge of the costal pleura with the handle of the scalpel, and working in first a finger and then the whole hand until the pleura is entirely free.   In passing the hand into the pleural cavities protect the back of it, especially if the ribs have been cut through, by folding the skin-flap in over the edge of the ribs.

If desired, the lungs can be drawn forward, examined over their whole extent, even incised, and then replaced until the heart has been removed.   In the connective tissue of the *anterior mediastinum* there is almost always a certain amount of emphysema due to the removal of the sternum.   Emphysema due to laceration of lung-tissue is more marked in the upper half of the mediastinum, and usually extends up into

the neck. The thymus gland attains its full development at the end of the second year, after which time it usually gradually atrophies.

Opening of the Pericardium.—To open the pericardium, seize the sac near the middle with fingers or forceps, snip through the wall with knife or scissors, and with either instrument cut upward to where the pericardium is reflected over the large vessels, downward to the lower right border, and lastly to the apex. By gently raising the apex of the heart the amount of fluid in the pericardial cavity can be seen. The normal amount is about a teaspoonful, but it may be increased to 100 c.c. in cases where the death-agony is prolonged. Pericardial adhesions should be broken through with the fingers. If this is impossible, the heart must be incised through the pericardium.

External Inspection of the Heart.—Determine first the position, size, and shape of the heart, and the degree of distention of the different parts. The right ventricle and both auricles are usually distended with blood, which may be fluid as in death from suffocation or more or less coagulated. The left ventricle is contracted and empty unless the individual has died from paralysis of this part of the heart, when it will be found distended with blood (condition of greatest diastole).

Opening of the Heart.—The heart may be opened *in situ* or after removal from the body. Except in certain cases, to be spoken of later, it usually will be found advisable to remove the heart before making any incision into it, for the reason that it can be more perfectly opened after removal, especially by beginners, and the danger of contaminating any bacterial lesions of the valves is lessened.

To *remove the heart*, grasp it gently near the apex with the left hand, supporting it further, if necessary, by one or two fingers placed above the coronal suture, and lift the whole heart vertically upward. Then cut its vessels from below upward with the knife held transverse and oblique. Divide in turn the inferior vena cava, the pulmonary veins on both sides, the

superior vena cava, the pulmonary artery, and the aorta. Go deep enough to remove the auricles entire, but avoid injury to the underlying eosphagus.

For making the incisions to *open the heart* either a long, slender-bladed knife or long, straight scissors may be used. The heart should be placed on a board with its anterior surface up. The right auricle is opened by cutting from the orifice of the inferior vena cava into that of the superior, and from the latter into the auricular appendage. The first incision to open the right ventricle is made through the tricuspid valve and the wall of the ventricle along the under surface
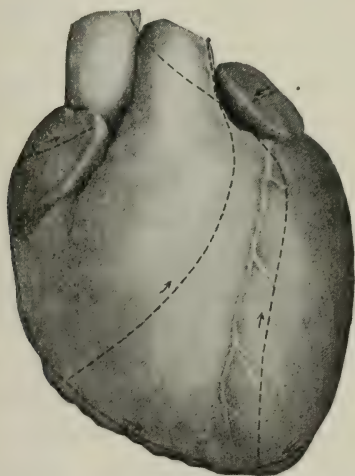
FIG. 158.—Heart, showing incisions.

of the right border of the heart. It should be carried to the end of the ventricle, which does not reach quite to the apex of the heart. The second incision begins about the middle of the first, just above the insertion of the anterior papillary muscle (which should not be cut), and is carried through the pulmonary valve well over on the left side along the left border of a narrow, projecting ridge of fat-tissue usually present, so as to pass between the left anterior and the posterior segments of the valve.

The left auricle is opened in a manner similar to the right by incisions joining the four orifices of the pulmonary veins and extending into the auricular appendage.

The first incision into the left ventricle is through the mitral valve along the left border of the heart (*i.e.* the middle of the external wall of the left ventricle), between the two bundles of papillary muscles, to the apex of the heart. The second incision begins at the termination of the first at the apex, and is carried up close to the interventricular septum, parallel to the descending branch of the anterior coronary artery and about 1 cm. from it. The upper portion of the incision should pass midway between the pulmonary valve and the left auricular appendage. Ordinarily, one of the aortic cusps is divided, but this may be avoided, if desired, by dissecting away to some extent the pulmonary artery from the aorta and carrying the incision well over to the right between the right posterior and anterior valve-segments. As each auricle is opened the blood and clots it contains should be carefully removed and the auriculo-ventricular valves carefully inspected from above. In certain cases—as, for instance, extreme stenosis—it may be preferable not to cut through the valve, but to begin the incision in the ventricular wall below the valve. The ventricular cavities should in like manner be freed from clots and the valves closely inspected. The coronary arteries should always be opened by means of small, narrow-bladed, probe-pointed scissors as far as they can be followed. The examination of the descending branch of the anterior artery is especially important. The posterior coronary is best opened by placing the tip of the left fore finger in the aorta over the orifice of the artery, and cutting from without in toward the finger-tip until the vessel is reached, when it can easily be slit up. In this way injury to the aorta is avoided. Sometimes it is advisable to examine the coronary vessels by means of multiple transverse sections instead of by slitting them longitudinally: the danger of disturbing emboli or thrombi within the lumen is less.

In cases of more or less sudden death with symptoms of asphyxia the pulmonary artery should always be opened *in*

situ before removal of the heart, in order to examine for possible emboli, because they often lodge just at the point where the vessels are severed in removing the heart and lungs, and easily may slip out unobserved. The simplest operation is to thrust a sharp-pointed scalpel through the artery just above the valve on the left side in the line of incision already described, and to cut upward until the branches to the right and left lungs are reached. If desired, this incision may be extended down through the pulmonary valve and the ventricular wall along the line given for the second incision in the right ventricle.

The water-test for the competence of the valves of the heart is not very reliable, especially for the auriculo-ventricular valves, and is not so much used as formerly. Inspection and measurement of the valve after the heart has been opened will usually enable one to judge fairly accurately concerning the degree of competence. Before applying the test to the aortic valve the first incision into the left ventricle must be made and the cavity freed from clots, so that no obstruction will exist below the valve. Then the heart is to be held so that the aortic valve is perfectly horizontal, and water poured in from above to float the cusps out. If competent, they should keep the water from flowing through. If, however, in holding the heart the normal relations of the valve and the surrounding parts are not maintained, the valve may leak. A second source of error is that the water may escape through the coronary arteries, branches of which have been cut in opening the ventricle. In testing the mitral valve the left auricle is first opened and the clots removed, so as to expose the upper surface of the valve. Then the nozzle of a syringe is introduced through the aortic valve and water forced in so as to float the mitral curtains up. The test, however, is very unreliable, because the parts cannot be placed under natural conditions.

The pulmonary and tricuspid valves can, of course, be tested by methods similar to those already described.

Increase or diminution in the size of the heart is best determined by weighing the organ after the removal of the clots.

In certain cases, however, and in special investigations measurements of different parts of the heart are desirable. Roughly, the heart is the size of the individual's fist.

The following *weights* and *measurements* are taken from *Nauwerck's Sectionstechnik:*

Weight of the heart averages in men,   300 gr. } *Orth.*
    "   "   "   "   " women, 250 "

Krause gives the average weight of the heart as 292 gr.

Relative weight of heart to body in men,   1–169 } *Krause.*
   "   " " " " " " women, 1–162

Length of heart in men,   8.5–9   cm. } *Bizot.*
   "   "   women, 8.0–8.5 "

Circumference of heart at base of ventricles, 28.8 cm.   (*Sappey*).

Thickness of wall of left ventricle, 1.1–1.4 cm. } *Krause.*
   "   "   right   "   0.5–0.7 "

Thickness of wall of left ventricle (without trabeculæ), 7–10 mm. } *Orth.*
   "   "   right   "   "   "   2–3   "

Circumference of mitral orifice,   10.4 (W.), 10.9 (M.) }
    "   " tricuspid "   12.0 (W.), 12.7 (M.) }
    "   " aortic   "   7.7 (W.), 8.0 (M.) } *Krause.*
    "   " pulmonary orifice, 8.9 (W.), 9.2 (M.) }
    "   " ascending aorta,   7.4 cm.
    "   " pulmonary artery, 8.0 cm. (*Buhl*).

The directions given for the removal and opening of the heart apply only when the organ is normal or contains lesions within itself which are not in continuity with any of the vessels entering into it. In aneurysm of the ascending aorta, in thrombosis of a vena cava, and in a number of different lesions connected with the heart or with the vessels given off from it, it is important to examine these vessels and to open them while they are still in continuity with the heart. For this purpose it is often necessary or advantageous to remove the thoracic organs in one piece, so as to be able to examine the central circulatory apparatus in continuity from the front and back before disturbing any of its relations. This is done by cutting across the trachea and adjoining tissues as high in the neck as necessary or possible, and dissecting them free from the cervical vertebræ and the first ribs. Then by drawing the trachea

and surrounding tissues forcibly forward the aorta and overlying organs can be easily stripped from the vertebral column as low as the diaphragm. The left hand is now placed around the lower end of the pericardial sac, the aorta, and the eosophagus just above the diaphragm, and the vessels are severed by cutting between the hand and the diaphragm.

More space for the examination *in situ* of the vessels at the base of the neck can be obtained by freeing the clavicles from all attachments above and to the first ribs and drawing them forcibly outward; this operation will be found especially useful in following up the subclavian vessels.

Removal of the Lungs.—Pleural adhesions have already been spoken of. If the base of the lung is adherent to the diaphragm, it is usually advisable to remove the latter with the lung by cutting through its insertion into the ribs. According to Orth, there is less danger of wounding the abdominal organs if scissors be used for the performance of the operation. After the lung is free it is drawn forward out of the pleural cavity, and the root of it is grasped from above downward between the separated fingers (first and second or second and third) of the left hand. The lung, thus resting in the palm of the left hand, is first drawn downward toward the pubes until the primary bronchus is divided by a nearly vertical incision above and behind the left hand. Then the lung is lifted vertically upward, and the rest of its attachments cut in the same direction from above downward by the knife held transverse and flat, so as to avoid injuring the esophagus and aorta.

The procedure is the same for both lungs. Once in a great while the apex of a lung will be found so firmly adherent by dense scar-tissue that it can be freed only by using the knife.

The primary or main incision into a lung is a long, deep cut from the apex to the base and from the convex surface to the root, slitting the primary bronchus, and thus not cutting it off from its branches to the upper and lower lobes (Fig. 159). To incise the left lung, place it with its inner or median surface and root downward on a board and with its base toward the operator. The left thumb steadies the lower

lobe; the first finger reaches between the two lobes almost to the primary bronchus; and the rest of the fingers should hold the upper lobe.

The right lung is most easily incised by placing it in the same position, but with the apex toward the operator; in other words, always place the anterior edge of a lung beneath

FIG. 159.—Method of incising the lung (Nauwerck).

the palm of the hand. Some prefer to place each lung on its lower or diaphragmatic surface for incision. The right middle lobe is incised separately by a cut extending transversely in its greatest diameter.

The bronchi and blood-vessels should be opened up for some distance with small probe-pointed scissors—as a rule from the surface of the section—cutting through the overlying lung-tissue. In some cases, however, it is best to open up both the blood-vessels and the bronchi from the outside

of the lung before incising it. The order to follow is vein first, then artery, and finally the bronchus.

Secondary cuts into the lung are to be made parallel to the main incision.

The peribronchial lymph-nodes should be incised from the outside of the lung.

Organs of the Neck.—The operation of the removal of the organs of the neck is greatly facilitated if it is possible to continue the primary skin-incision up to the chin. In other cases dissect the skin from the larynx and muscles of the neck as far up as possible. In like manner free the muscles, esophagus, and trachea from their attachments laterally and posteriorly. Then allow the head to drop well back over the end of the table, and pass a long, slender-bladed knife up between the skin and the larynx, just behind the symphysis of the lower jaw, until the point of the knife appears beneath the tip of the tongue. From this point the knife is carried with a sawing motion down first one ramus of the jaw and then the other, dividing laterally the glossal muscles as far back as the posterior pharynx. The knife is next carried up behind the esophagus, and the posterior wall of the pharynx divided as high as possible. Pass the left hand up inside of the neck and draw down the tongue. Then cut the attachments of the soft to the hard palate, carrying the knife well out so as not to injure the tonsils. Any remaining attachments are usually easily severed by pressing the tongue first to one side and then to the other, and cutting close to the roof of the pharynx.

Each lobe of the thyroid gland is to be incised in its greatest diameter.

Next cut through the middle of the uvula and examine all of the pharynx removed. Incise the tonsils vertically. The esophagus is to be slit in the median line posteriorly; if it is normal, the larynx and trachea are then slit in the posterior median line also, thus splitting the esophagus in two.

The Abdominal Cavity.—The order of removal of the abdominal organs varies with different operators, and under varying circumstances with the same operator. The gastro-

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intestinal tract, including the liver and pancreas, may be removed before or after the genito-urinary tract. The spleen as an organ by itself is often the first to be removed. The early removal of the liver is occasionally advantageous for the sake of the additional space obtained for the examination of the other organs. It is well to practise the different methods of procedure, so that in a difficult case the best may be selected, because the examination of the abdominal cavity, especially in cases of extensive disease with numerous adhesions, is often one of the hardest tasks in post-mortem technique. As a rule, it is best to follow the usual order as long as possible, gradually removing the more or less normal or uninvolved organs. Occasionally it may be advisable to remove the organs *en bloc*, so as to be able to approach the problem from all sides.

In all cases of acute peritonitis it is best before removing any organ to search for the source of the infection, paying particular attention to the vermiform appendix, to the gastrointestinal tract, and, in females, to the pelvic organs.

The order of removal of the abdominal organs adopted in this book for the majority of cases is that which seems the simplest and most natural—namely, to remove first the spleen as an organ essentially by itself; secondly, the gastrointestinal tract, including the pancreas and liver, which forms the upper layer; thirdly, the genito-urinary tract or middle layer, leaving the circulatory tract, the lowest layer, to be opened and inspected *in situ*. If, however, it proves necessary to open a part of the gastro-intestinal tract *in situ*, it will be neater perhaps to remove the kidneys and spleen first. Occasionally at private autopsies it may be unnecessary to examine the intestinal tract; under these circumstances it is important to be able to get at the different organs without taking out the intestines.

The Spleen.—As a rule, the spleen can easily be drawn forward from its bed behind the fundus of the stomach, beneath the diaphragm, and lifted on to the lower edge of the ribs on the left side without cutting its vessels. The organ is then to be incised in its greatest diameter while thus firmly fixed between

the left hand and the ribs; or the vessels may be cut close to the hilus and the spleen incised after being placed on a board.

In cases of adhesion to the diaphragm the spleen must be handled carefully while the fibrous attachments are torn or cut through, for the capsule is easily ruptured. Occasionally it is advisable to cut out with the spleen the portion of diaphragm attached to it.

The important anatomical structures to be noted in the macroscopic examination are the capsule, trabeculæ, blood-vessels, lymph-nodules, and pulp. The weight of the spleen, according to Orth, varies from 150 to 250 grams. The average weight is put at 171 grams. The spleen measures 12 × 7.5 × 3 cm.

The Gastro=intestinal Tract.—The first step is to examine externally, more or less carefully according to the clinical symptoms, the whole tract from the stomach to the rectum, if it has not already been done at the primary inspection of the peritoneal cavity. The main points to notice are distention or contraction of the intestines, injection of the blood-vessels, thickening of the wall, especially in the lower part of the ileum, adhesions, exudations, etc. Inspect the mesentery, its length, the amount of fat, and the size of the lymph-nodes; incise the latter to determine color and consistence. Examine the mesenteric vessels if any evidence of infarction of the intestine is noticed. The portal vein and its branches should be opened up *in situ*, in all cases of abscess of the liver or of secondary deposits in it of malignant growths, before the gastro-intestinal tract is removed. As a rule, it is not necessary to open any part of the gastro-intestinal tract *in situ*. The operation can be performed much more neatly at the sink. The duodenum is often opened for the sake of investigating the flow of bile from the gall-duct, but except in cases of jaundice the operation must be looked upon largely as a physiological experiment.

Free the *omentum* from the transverse colon by putting it on the stretch and dividing it with the knife close to the colon. Then begin the removal of the large intestine by drawing the sigmoid flexure forcibly forward and cutting the mesocolon

close to the gut, first down to the rectum, then upward to the transverse colon. Free the latter by dividing the two folds of the lesser omentum, if not already cut through, which unite it to the stomach. The ascending colon is to be freed in the same manner as the descending portion. Care should be taken not to injure the appendix. If the lower part of the sigmoid flexure be now stripped upward a short distance with the fingers, so as to force the intestinal contents out of the way, the gut can be divided just above the rectum without fear of the feces escaping.

Place the freed intestine in a pan or pail, and as the small intestine is divided from its mesentery deposit it in the same receptacle. To remove the small intestine, begin at the cecum, and, while lifting the ileum with the left hand strongly enough to keep the mesentery constantly tense, cut the latter close to the intestine by playing the knife easily backward and forward across it with a fiddle-bow movement. Continue the operation until the duodenum is reached. The mesentery can now be dissected from the duodenum and removed, or the mesentery, duodenum, pancreas, and stomach can be removed in continuity with the intestine by carefully dissecting them off the underlying structures. The operation is perhaps more easily accomplished by freeing the organs from below upward. First cut down through the diaphragm and free it around the esophagus. Then separate the stomach from the liver by means of the thumb and fingers of the left hand in such a way as to put on the stretch the vessels of the hepato-duodenal ligament. These vessels (hepatic artery, common bile-duct, and portal vein) are then carefully divided in the order named. As each vessel is cut the character of its contents should be observed to see if anything abnormal is present.

The mesentery, if still present, the duodenum, the pancreas, and the stomach, are now to be dissected carefully away from the underlying vessels from below upward until the esophagus is reached. This may be constricted by the fingers at any point desired, and cut across without danger of the gastric contents escaping and without the necessity of tying. In certain cases of hemorrhage from the stomach associated with cirrhosis of the

liver it is important to remove the esophagus in continuity with the stomach, because in these cases the hemorrhage usually takes place from dilated esophageal veins.

The *stomach* and *intestines* are now to be opened at the sink by means of the enterotome, the colon along one of its longitudinal muscular bands, the small intestine along its mesenteric attachment, because the most important lesions usually occur opposite this line in the lymph-nodules and Peyer's patches. The stomach is opened by many along the greater curvature; others, however, prefer to cut along a line 3 cm. from the lesser curvature, on the ground that better museum preparations are thus obtained. In case any tumor or focal lesion is perceived from the outside, it is advisable to cut the stomach, if possible, in such a way as to leave the pathological part uninjured.

Whenever jaundice is present the duodenum must be opened *in situ* in order to examine the bile apparatus in continuity, so as to determine whether the coloring is due to obstruction of the hepatic or common bile-duct, or is of so-called hematogenous origin.

To open the *duodenum* make a transverse fold in the anterior wall and incise with the scissors. Continue the longitudinal slit thus made up as far as the pylorus and down to where the duodenum passes beneath the mesentery. Notice the contents of the duodenum and their color both above and below the opening of the bile-duct. The ductus choledochus usually opens in common with the ductus pancreaticus on the posterior wall of the duodenum a little below the middle of the head of the pancreas, at a point marked by a small papilla which can easily be recognized by putting the mucous membrane on the stretch transversely. Press first on the common duct gently and in the direction of the papilla, watching the opening to see if any obstructing material is forced out. Pressure is then to be made on the gall-bladder to see if its contents also will flow. If necessary, the common duct and its branches are to be opened *in situ*. In certain cases the ductus pancreaticus is likewise to be opened up.

Several cross-sections of the *pancreas* are usually better than one in the greatest diameter, because the duct is left in a better condition for slitting up if necessary. The weight of the pancreas varies from 90 to 120 grams (Orth). It measures 23 × 4.5 × 2.8 cm.

The Liver.—The liver is usually the last organ of the gastro-intestinal tract to be removed. This is ordinarily done by lifting up the right lobe and freeing it from all attachments as far as the vertebral column: the right lobe is then lifted and placed on the edge of the ribs on the right side, while the left lobe is elevated and freed. If the diaphragm is firmly adherent, remove it with the liver. The incision to display the liver is a long deep cut passing through the right and left lobes in the greatest diameter of the organ.

In a good many cases it is very convenient to remove the liver at the beginning of the special examination of the abdominal cavity, because more room can be obtained for the investigation of the other organs. This latter fault can to some extent be obviated by cutting the diaphragm on the right side and allowing the liver to slide forward somewhat into the right thoracic cavity.

There can be no objection to the removal of the liver when jaundice is not present or when the liver is not connected by continuity with the lesion of some other organ (pylephlebitis, malignant growth extending through portal vein or along bile-ducts, etc.).

The operation is performed as follows: Pass the left hand in between the diaphragm and the right lobe and push the liver forward out of the right hypochondrium. Incise it deeply in its greatest diameter through the left and right lobes. Next free the gall-bladder from its bed by means of the fingers, and cut it off near the ductus hepaticus after compressing its lower end. It can then be opened lengthwise and washed without danger of discoloring the liver or other organs. The liver is now to be grasped by placing the thumb on the under surface of the liver and the fingers in the incision. Elevate the organ, and, while carefully watching, cut through

the hepato-duodenal ligament, which includes the blood-vessels and the ductus hepaticus. The ligamentum hepato-gastrium, the inferior vena cava, the suspensory ligament, the ligamentum coronarium, and the tissue between the inferior surface of the liver and the upper end of the kidney follow next: the adrenal is to be left on the kidney, and the diaphragm ought not to be injured.

Even in the ordinary way of removing the liver the organ will be found much easier to handle if the usual incision is made *in situ*, so as to furnish a hold for the left hand.

Other cuts into the liver are best made parallel to the primary one.

Orth gives the weight of the liver for adults as varying from 1000 to 2000 grams. The average weight is usually put at 1500 to 1800 grams.

The liver measurements are as follows:

Length from right to left,	25–32	cm.
Width of right lobe,	18–20	"
Width of left lobe,	8–10	"
Vertical diameter of right lobe,	20–22	"
Vertical diameter of left lobe,	15–16	"
Greatest thickness,	6– 9.5	"

The Kidneys and Adrenals.—If the adrenals are to be removed with the kidneys, it is necessary to cut first to the inside, and secondly above the adrenal, and then to make from the outer end of the second cut a curved incision along the outer convex border of the kidney through the peritoneum and the perinephritic fat-tissue. The left hand is to be inserted into the cut, the mass of tissue drawn forcibly forward, and the vessels divided as close to the aorta as possible, so that the renal vessels may be slit up and examined in connection with the renal lesions. The adrenal should be incised crosswise. The kidney is to be held firmly in the left hand between the thumb and fingers while a longitudinal incision is made from the convex border to the hilus. As a rule, it is better to shell it out of its investing fat-tissue before incising it.

It will often be found convenient to make simply the curved incision above given, to shell the kidney out of its fat-capsule, and then to divide its vessels, leaving the adrenal behind to be incised *in situ* or removed separately.   As a rule the left kidney is removed first.

In all cases in which the bladder is involved in pathological changes in common with the kidneys the whole urinary tract should be removed intact, so that the lesions may be examined in continuity.   For this reason it is a good plan to open up the pelvis of the kidney and the ureter from the primary incision, in order to see if any lesion is present before dividing the ureter.

If it is desired to remove the kidneys before the intestines, the latter must to some extent be freed from their normal attachments.

The splenic flexure of the colon is first to be drawn forcibly forward and its attachments divided where they hide the left kidney.   If the ureter is to be taken out also, it is best to free the whole of the descending colon from its mesocolon.   Then the colon and the coils of small intestine are drawn over to the right side of the body, so as to leave the left kidney and adrenal exposed.   They are then removed in exactly the same manner as already described.

To remove the right kidney the hepatic flexure must be freed from over it.   If the ureter is to be taken out, the descending colon and the cecum are dissected from over it.   The right adrenal is firmly attached to the under surface of the liver, and must be carefully dissected from it by turning the latter upward.

If the urinary tract is to be removed in continuity, each ureter is dissected down to the brim of the pelvis, and then left with its kidney attached until the pelvic organs have been taken out.

After the kidney has been incised the capsule is to be stripped off, at least in part, so that the appearance of the surface of the kidney and the presence or absence of adhesions between the capsule and the renal tissue can be determined.

The points to be noted in the macroscopic examination of the kidney are size, consistence, and, on section, color, relative proportion of cortex to pyramids, and thickness of each;

finally, the normal markings of the kidney, including blood-vessels, glomeruli, convoluted and straight tubules of cortex, collecting tubules of pyramids.

The average weight of the kidney is 150 grams. The left kidney is always 5 to 7 grams heavier than the right (Orth). A kidney measures 11–12 × 5–6 × 3–4.5 cm. The cortex measures in thickness 4–6 mm. The relation of the cortex to the medulla is 1 to 3.

The Pelvic Organs.—The pelvic organs are most easily and neatly removed by stripping the peritoneum from the pelvic wall with the fingers. Begin over the bladder and extend down the sides of the pelvis until the fingers meet beneath the rectum. Brace the backs of the hands laterally on the brim of the pelvis and lift the fingers forcibly upward; this movement will free the pelvic organs cleanly from the sacrum, and leave them attached only anteriorly at the rectal and genital openings, and posteriorly by the peritoneum and the vessels at the brim of the pelvis.

Anteriorly, the attachments may now be divided with the knife at whatever point seems advisable, ordinarily close to the pubes just anterior to the prostate (or through the urethra and vagina in females) and through the lower end of the rectum. Posteriorly, cut through the tissues at the brim of the pelvis, taking care not to cut the ureters if the kidneys are still attached to them. The *rectum* is to be opened with the enterotome along the posterior wall, and the inner surface thoroughly washed off so as to avoid soiling the other organs.

To open the *bladder* in males, especially if the penis has been removed in continuity with it, incise with the scissors a transverse fold in the anterior wall of the fundus, and carry the incision through the urethra and along the dorsum of the penis. To accomplish the latter act perfectly the *penis* must be firmly stretched by having an assistant pull at the frenum while the bladder is held fixed by the operator.

In females it is usual to enter the scissors into the bladder through the urethra and to cut through the middle of the anterior wall of the fundus.

In males the *rectum* should be dissected from the bladder, so as to lay bare the vesiculæ seminales and the prostate, which are examined by means of several transverse incisions.

In females, if the bladder is normal, the *vagina* is incised in the anterior wall through the middle of the bladder. Or the vagina may be incised laterally until the cervix is reached, and then the cut be carried up to the median line.

The *uterus* is incised in its anterior wall from the cervix to the fundus. From the upper part of this incision secondary incisions are carried out on each side to the orifices of the Fallopian tubes.

The *ovaries* are incised in their greatest diameter, from the convex border to the hilus. Weight of ovaries, 7 grams.

The *testicles* can readily be examined without external injury to the scrotum by cutting underneath the skin over the pubes down to the scrotum on either side of the penis, and shoving the testicles up through the incision. Cut carefully through the overlying tissues until the cavity of the tunica vaginalis is opened. Remove the testicle by svering the cord. The incision to display a testicle should be in the long diameter, beginning on the side opposite the epididymis and extending through into it. Weight of testicles, 15–24.5 grams. In cases of tuberculosis of the testis and epididymis it is advisable not to cut through the cord, but to remove the testicles and cords with the bladder, so that the whole genital tract may be examined in continuity and the associated lesions in the vesiculæ seminales demonstrated, if present.

The *penis*, or at least the larger portion of it, can be removed in connection with the bladder by continuing the primary body-incision out to about the middle of the dorsum of the penis, which is then to be freed from the investing skin and divided just posterior to the corona. It is next dissected back to the pubic arch, and freed from it partly by cutting from without, partly from within, the pelvis, until the penis can be passed underneath the arch into the pelvis. Other methods are to cut through the symphysis, which can then readily be sprung apart by swinging one of the legs out in a horizontal plane, or

even to saw out a small section of bone including the symphysis, so as to have more room for freeing the attachment of the penis and for removing it.

The structures now remaining in the abdominal and thoracic cavities which require examination are the large blood-vessels, the thoracic duct, the celiac ganglion, and the retroperitoneal lymph-nodes. The *inferior vena cava* and its branches are first examined (especially in all cases of pulmonary embolism) by slitting them with scissors along the anterior wall. If it is necessary to follow the iliac vessels into the thigh, it will be found easier in sewing up if the primary abdominal incision is continued off to the side in question, thus giving a single though curved incision.

It is sometimes advisable to open up the inferior vena cava and its branches before removing the pelvic organs, so that thrombi extending into the pelvic vessels may be examined before they are disturbed.

The *semilunar ganglia* lie on the aorta, around the celiac axis, above the pancreas.

The *thoracic duct* lies behind and to the right of the aorta. In the thorax it is most easily found by dissecting on the right side between the aorta and the azygos vein. The receptaculum chyli lies to the right and behind the aorta upon the second or third lumbar vertebra. Examination of the thoracic duct is of especial importance in cases of tuberculosis of the intestine and mesenteric lymph-nodes with secondary miliary tuberculosis.

The *aorta* is to be opened *in situ* along the anterior wall throughout its whole extent, and the iliacs as far as the femoral ring.

Besides the brain, the spinal cord, and the thoracic and abdominal organs, it is often necessary to examine or remove for study other portions of the body that are affected by disease. A little ingenuity will enable one in appropriate cases to get at almost any part desired.

A view of the marrow in a long bone is most easily obtained in the femur by extending the body-incision down over one of

the thighs, dissecting the muscles away, making two transverse, oblique, converging cuts with a saw, 3 to 5 cm. apart and extending about one-third through the shaft, and then chiseling off the included piece of cortical bone.

In tuberculosis of the spine it is quite easy to remove any part, or even the whole, of the vertebral column, including the pelvis and portions of the femurs, without other incisions than the one from the neck to the pubes, with extension down the thighs in case parts of the femurs are to be taken out. Divide the ribs a few centimeters from the vertebral column on each side of the portion that is to be removed, cut through intervertebral disks both above and below it, and then carefully dissect it free, taking great care not to button-hole the skin.

Removal of the Brain.—The incision into the scalp should begin from 1 to 2 cm. behind the right ear, near its lower border, at the edge of the hair, and extend over the vertex of the skull to a corresponding point behind the left ear. The cut is most easily made by thrusting a small narrow-bladed scalpel, with its back toward the calvarium and its point toward the vertex, through the skin behind the ear and shoving it along in the desired direction. By making the incision in this manner the hair is not cut, but simply parted. The anterior flap should be stripped from the calvarium and the temporal muscles by putting it on the stretch and dividing the loose connective tissue holding it by sweeping strokes of the scalpel nearly as far forward as the orbits. After a part of the flap has been freed it is often possible to strip the rest without using the scalpel. For the posterior flap, which should be removed back as far as the occipital protuberance, the scalpel nearly always has to be used.

If the hair is long, the anterior portion can be rolled into the anterior flap over the face and thus protected. The posterior portion is gathered at the nape of the neck, and then a towel is wrapped tightly around the head and neck, extending from the line where the flaps are reflected down to the shoulders, and is pinned over the lower part of the

forehead. In this manner the hair is perfectly protected from being soiled and ample room is left for work.

Of the two methods of opening the skull, the circular and the wedge-shaped, the former makes the better museum preparation, but the latter is in greater use in this country, and has the advantage of rendering the calvarium less likely to slip out of place after the head has been sewed up.

The wedge-shaped incision consists of three cuts, which should be outlined on the periosteum of the skull with a scalpel. The first cut begins just above and behind the left ear, and is carried over the forehead just back of the edge of the hair or over the frontal eminences to a corresponding point above and behind the right ear. The two other cuts begin at each end of the first incision, forming there an obtuse angle, and are carried back to meet in the median line behind at an angle of about 160° a little in front of the occipital protuberance. The temporal muscle on each side is now to be scraped back from the line of incision out of the way of the saw, but is not to be cut off. The holder, if one is used, is attached with a foot in each obtuse angle in the temporal region. If a holder is not employed, the head is best steadied by hands on the calvarium and face. Use towels or cloth to prevent slipping.

Start the incision with the saw over the forehead and extend it back along the line marked out. It is best not to carry the incision clear through the inner table of the bone, for two reasons: first, on account of the danger of injuring the brain-substance; secondly, because if the inner table or a part of it is cracked through with a chisel and hammer, it can be done without injuring the underlying tissue, and the irregular over-lapping fragments of bone thereby formed serve afterward for holding the calvarium firmly and steadily in place.

After sawing along the lines marked out, insert a chisel in the frontal region, and with a quick, sharp blow crack through the rest of the inner table. In like manner insert the chisel in the middle of the other incisions and free the calvarium posteriorly. To remove the calvarium insert the chisel end of the hammer in the incision in the frontal region, and press down

with the left hand while swinging the handle around in a horizontal plane.

By means of the powerful purchase obtained the calvarium is easily started. Then catch the hook of the hammer over the calvarium and strip it off. If the dura is adherent to the calvarium, it may be freed by using the point of the closed enterotome to pry it off.

In young children, and sometimes in old people, it is necessary to remove the dura with the calvarium. To do this, cut through the dura with the point of a scalpel along the lines of incision in the skull; then cut the falx cerebri in the median line, both anteriorly and posteriorly.

An infant's skull is best opened by cutting with a pair of scissors through the dura along the sutures (in the longitudinal fissure on each side of the falx) well down to the floor of the skull. This gives five bone-flaps which may be turned out like the petals of a flower, leaving the brain uninjured. It is often necessary to cut half of the base of each flap in a horizontal line to aid its being turned out. The falx cerebri must of course be divided anteriorly and drawn back before the brain is removed. In sewing up, the bone-flaps are turned in over a bag of sand or sawdust filling the cranial cavity, and are kept perfectly in place by the skin.

In a case of fracture of the skull no cracking with hammer and chisel is allowable; the calvarium must be freed entirely by sawing. The calvarium should be examined at the time of removal.

The next step is to inspect the dura. Under normal conditions it is not tense in the frontal region, but can be picked up with the forceps or fingers. If the dura is not thickened the convolutions normally should be visible through it. The longitudinal sinus is opened with knife or scissors and its contents examined. Pacchionian granulations are not infrequently found projecting into it.

To remove the dura, cut through it with scissors or knife along the same lines in which the calvarium was sawn. Turn back each half of the dura and examine the surface of the

convolutions and the inner surface of the dura. The convolutions should be distinct and rounded, not flattened with obliteration of the gyri, as occurs when there is internal pressure.

The Pacchionian granulations are situated along the longitudinal fissure and may grow through the dura and form depressions in the calvarium. There may be apparent adhesions between the dura and pia due to veins passing from one to the other. The dura is still further freed by seizing the two halves anteriorly and lifting them up until the falx is tense at its insertion into the crista galli. Pass a knife in parallel to the falx, on the left side, with the edge forward, as far as the cribriform plate; turn it to the right and cut until the falx yields. Withdraw the knife in the same manner in which it was inserted. Next draw the dura back. It is usually more or less attached along the longitudinal fissure by Pacchionian granulations and by blood-vessels. These may be cut or torn through. Do not cut the dura posteriorly, but let it hang down.

To remove the brain, insert the two fore fingers, or the first and second fingers of the left hand, anteriorly between the dura and the frontal lobes, one on each side of the falx cerebri, and draw the brain gently back until the optic nerves are visible. Ordinarily, the olfactory nerves come away from the cribriform plate without trouble, but sometimes have to be freed with the point of the knife. With a long, slender-bladed knife divide the optic nerves as far forward as possible while holding the brain back with the left hand. Continue to draw the brain carefully back and divide the cranial nerves and the carotids. Then draw forward first the left, then the right temporal lobe, and cut the tentorium close to its attachment to the petrous portion of the temporal bone with a sawing motion, using the tip of the knife. Insert the knife at the side close to the squamous bone, and cut from there in toward the foramen magnum. Then cut the nerves given off from the medulla oblongata while supporting the convexity of the brain in the left hand.

Lastly, carry the knife as far as possible into the spinal canal, and divide the cervical cord by an oblique incision

from each side, severing the vertebral arteries with the same stroke. Better than a knife is the myelotome, because it gives a cross-section of the cord and allows more of it to be removed.
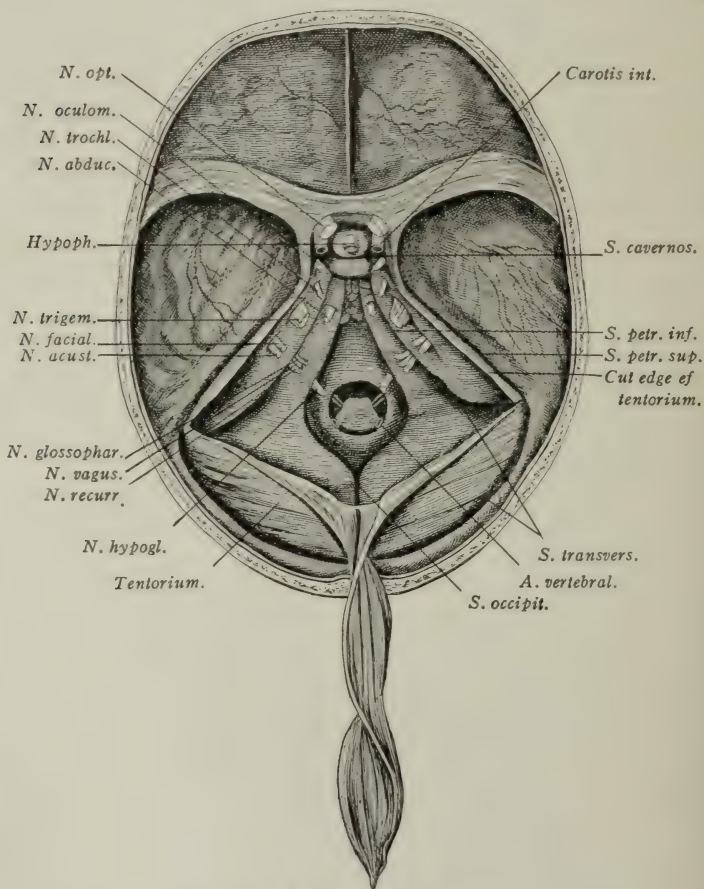
FIG. 160.—Base of skull (Nauwerck).

The brain is now to be removed by passing the first and second fingers of the right hand in on either side of the cord, and everting the brain while still supporting it posteriorly with the left hand.

Before proceeding to open the brain it is best to examine the base of the skull, particularly the dura, of which the sinuses should be incised, and the hypophysis cerebri.

If there is a suspicion of a fracture at the base, strip off the dura, so as to give a better opportunity for examination of the bone.

The brain should be weighed before it is dissected. The average weight in an adult male is 1358 grams; in an adult woman, 1235 grams.

External Examination of the Brain.—Place the brain with the base uppermost and with the cerebellum toward the operator. Examine first the pia and the cranial nerves, then the arteries, especially the middle cerebral and its branches on each side in the fissure of Sylvius, for it is here that emboli most frequently lodge. The pia bridging the fissure of Sylvius can sometimes be torn through, but usually has to be cut.

It is important, particularly in cases of obscure cerebral symptoms, to feel gently with the finger-tips all over the surface of the brain for any areas of increased density, because patches of sclerosis may in that way be found which might otherwise be overlooked.

By stripping off the pia—a procedure not often advisable—adhesions over pathological areas can sometimes be found pointing to the lesions beneath, but the pia should not be stripped from those portions which are to be examined microscopically. To remove the pia an incision is made on the median surface of each hemisphere just above the corpus callosum from one extremity to the other, and the pia stripped back first from the median and then from the convex surface. The stripping is done by means of the fingers, with occasional aid from the forceps.

Section of the Brain.—There are several methods of cutting up the brain, no one of which is particularly suitable to all occasions. That method must be chosen which is most fitted to the individual case and to the use to which the tissue is to be put.

The ideal method from a neuro-pathological standpoint would undoubtedly be to harden the brain entire, and then

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to make serial frontal sections thin enough for microscopical purposes through the whole organ. The nearest approach to this ideal method is to harden the brain entire in formaldehyde, a process occupying ten days to two weeks to make thin serial sections, to mordant the sections, divided if necessary into smaller pieces, in a chrome salt (preferably by Weigert's quick method), and then to carry through a number of series from the important parts for microscopical examination. By this means the relations of the various cerebral structures and of the pathological lesions can be perfectly preserved and studied. This method can be particularly recommended for tracing degenerations in the motor tract.

If there is a noticeable focal lesion, such as a tumor or hemorrhage, it should be so incised, generally frontally or horizontally, as best to show its relations to the important cerebral tracts and ganglia. In these cases also the best results are obtained by hardening the brain entire in formaldehyde, and later making serial sections for macroscopic study or for carrying through for histological purposes. In many cases, however, it is necessary or advisable to examine the lesions in the fresh state. For instance, if it be desired to study the neuroglia-fibers, it is positively necessary to cut out thin slices of fresh tissue and to fix them immediately in the proper solution. Often, too, the lesion cannot be or is not found except on fresh examination, or the clinician whose case it is desires to see at once the cause of certain symptoms. Under such circumstances the more ideal method must be sacrificed, and as much made out of the case as is possible in the condition in which it is left after the examination.

For the routine examination of the brain, to demonstrate its topography and to bring to light suspected or unsuspected lesions, probably no method is more generally used than Virchow's. The objection most often made against it is that the cerebral cortex is too much cut up. In case, however, it is desired to preserve the cortex or parts of it for microscopic purposes, the longitudinal incisions after the first may be omitted, and the cortical portion, after being separated from

the stem, may be cut in any way that seems advisable. In like manner, the brain-stem or any other part may be left uncut, and hardened entire in formaldehyde for histological purposes.

Virchow's Method.—The brain is to be placed on its base in the same position as one's own. Press the hemispheres
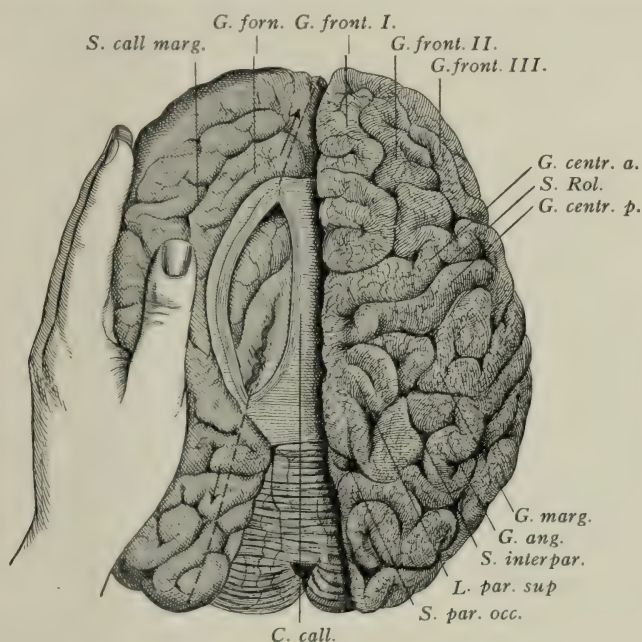
FIG. 161.—First cut in the brain (Nauwerck).

apart a little so as to expose the corpus callosum. Hold the left half of the cerebrum in the left hand with the fingers on the lateral aspect and the thumb in the longitudinal fissure. Then make an almost vertical incision with a long, slender knife through the roof of the left ventricle in its middle third, 2 to 3 mm. from the median raphe of the corpus callosum. The roof of the ventricle is to be slightly raised vertically by the thumb, so that the incision, which must not be too deep, may not injure the basal ganglia. The incision is to be continued into the anterior and posterior cornua. Then make a long

incision from one end of the above cut to the other, passing just outside of the basal ganglia at an angle of about 45°. Repeat the process on the right side, turning the brain half around. Next seize what remains of the corpus callosum and fornix in the middle, lift them, and cut through from below up, passing the knife through the foramen of Munroe. The parts are then turned back, exposing the velum interpositum and the choroid plexuses. By drawing back the velum interpositum the third ventricle is uncovered.

The corpora quadrigemina are exposed by cutting transversely the right posterior pillar of the fornix and adjoining brain-substance and carrying them over to the left. Each ventricle as it is opened is to be carefully inspected and any abnormal condition of its ependyma noted. The cortex is further divided on one side, and then on the other, by holding it in the left hand and making vertical straight sections from the upper angle of the previous cut into the convex cortex, allowing the sections to fall apart, so as to avoid touching and soiling the surface with knife or fingers. Each portion thus cut represents a prism. The incisions should go well into the cortex, but not so far as to separate the different pieces. The basal ganglia are examined by means of a number of frontal sections. For this purpose the left hand is placed palm upward underneath the brain, so that as each section is made over the tips of the fingers by one long stroke of the knife it falls forward, exposing a clean surface of which the two halves can be compared. An incision is next carried through the middle of the pineal gland, the corpora quadrigemina, and the vermiform process of the cerebellum, opening the aqueduct of Sylvius and the fourth ventricle.

Each half of the cerebellum is divided by a median horizontal section into halves, and these portions are still further subdivided by a series of cuts radiating from the peduncles.

In order to make sections of the pons and medulla the brain is folded together and turned over. Several cross-sections are then made with the left hand placed beneath as in sectioning the basal ganglia.

Before making the sections it is well to remove the basilar and vertebral arteries, especially if they are calcified.

In *Pitre's method* of dissecting the brain the lateral ventricles are opened as in Virchow's method. Then the pedunculi cerebri are cut squarely across, so as to remove the pons and cerebellum, and a longitudinal incision is carried down through the third ventricle, halving the cerebrum. Through each half of the cerebrum a series of six sections is then made parallel to
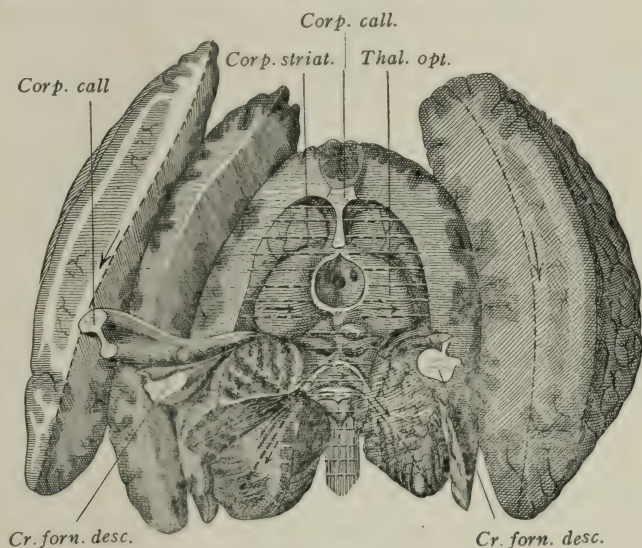
Fig. 162.—Section of the brain (Nauwerck).

the fissure of Rolando. The names of the sections and the important parts which they show are as follows:

1. The *pre-frontal section* through the frontal lobe, 5 cm. anterior to the fissure of Rolando, shows the gray and white substance of the frontal convolutions.

2. The *pediculo-frontal section* through the posterior portions of the three frontal convolutions shows the anterior extremity of the island of Reil, the lenticular and caudate nuclei, and the internal capsule.

3. The *frontal section* through the ascending frontal convolution, parallel to the fissure of Rolando, shows the optic

thalamus, the lenticular and caudate nuclei, the claustrum, the external and internal capsules, the anterior portion of the descending horn of the lateral ventricle, and the island of Reil.

4. The *parietal section* through the ascending parietal convolution shows portions of the same structures as the preceding, and a transverse view of the hippocampus.

5. The *pediculo-parietal section* through the parietal lobe, 3 cm. posterior to the fissure of Rolando, shows the tail of the caudate nucleus in two places and the posterior portion of the optic thalamus.

6. The *occipital section* through the occipital lobe, 1 cm. in front of the parieto-occipital sulcus, shows simply the white and gray matter of the occipital lobe. The cerebellum, pons, and medulla are incised in the manner already described.

Removal of the Spinal Cord.—The body is to be placed face downward, with the head over the end of the table and a block under the chest. The incision is made over the spinous processes from the occiput to the sacrum. Dissect the skin and muscles back on each side, so as to leave the vertebral laminæ as bare as possible. The laminæ may be cut through by means of several instruments, of which the double-bladed saw (Luer's rachïotome) is perhaps the safest, at least for beginners. The single-bladed saw with rounded end is also very useful and can be thoroughly recommended. The operation can be done most quickly by biting off the spinous processes with the heavy bone-forceps and cutting through the laminæ with chisel and hammer, but there is greater danger of injuring the cord.

The numerous artifacts in the cord, reported as neuromata and heteroplasia even within very recent times by competent pathologists, would seem to indicate that the need of careful and delicate technique in the removal of the spinal cord is not yet fully appreciated.

The laminæ should be sawn nearly or entirely through in a line with the roots of the transverse processes from the third or fourth lumbar vertebra to the cervical region. The arches of the cervical vertebræ are best divided with a heavy bone-cutter, because they cannot be easily sawn, and there

is sufficient room here for the point of the bone-cutter without danger of their pressing on the cord.

It is important to strike the outside limits of the spinal canal, so as to get as much room as possible for the removal of the cord. Test if the sawing be deep enough by the mobility of the spinous processes. If necessary, they can be freed by means of the hatchet-chisel and a hammer in the same way that the calvarium is loosened.

As the cord reaches only to the second lumbar vertebra, cut through between the third and fourth, free with the heavy bone-cutter the lower end of the row of the spinous processes, which are held together by their ligaments, and strip them up to the neck; then cut through the cervical arches with the bone-cutter, taking care that the point within the canal does not come in contact with the cord.

The nerve-roots are to be divided with a sharp scalpel by means of a long cut on each side of the cord. Then cut across the dura and the nerve-roots at the lower end of the exposed canal, and, while holding the dura with forceps, carefully free the cord from below up with scissors or scalpel, taking care all the time not to pull or bend the cord, because in either way artifacts may be produced. Cut the cord squarely across as high in the cervical canal as possible, so that the remaining portion may be easily removed with the brain.

Lay the cord after removal on a flat surface and incise the dura longitudinally, first posteriorly and then in front. A series of cross-sections, usually 1 to 2 cm. apart, is made through the cord while supported on the fingers during the cutting, so that the cut surfaces shall fall apart. The different segments should ordinarily be left-attached to the dura, so that their position in the cord can easily be determined.

A diagnosis from the fresh, macroscopic appearances of the cord is often very difficult to make, according to the best authorities.

The Eye.—The contents of the orbit, including the posterior part of the eye, can be readily examined by chiselling off the roof of the orbit. The posterior half of the eye can be removed

by cutting around the eyeball with sharp scissors without changing the hold of the forceps on the sclera. If done quickly, the retina remains quite well spread out. The anterior half of the eyeball is to be propped in place by a plug of cotton dipped in ink or in a solution of permanganate of potassium.

The Ear.—The middle ear can be exposed by chipping off with a chisel its roof, which lies in the middle of the petrous
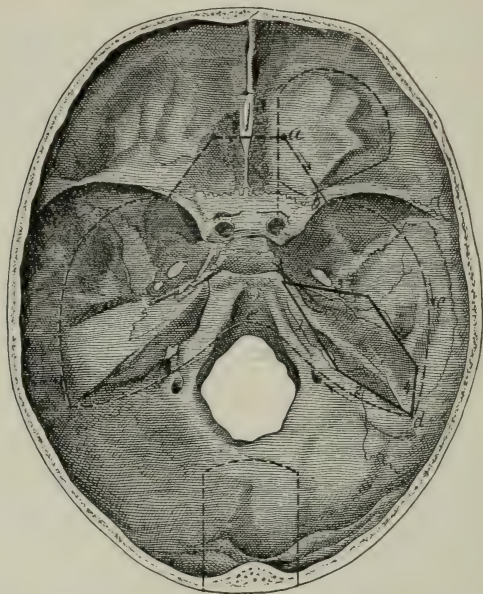
FIG. 163.—Base of skull, showing lines of incision for removing internal eye, etc. (Nauwerck).

portion of the temporal bone. The roof can also be very easily bitten off with the heavy bone-cutters. If, however, it be desired to examine the ear more carefully by means of a section through the external meatus and the middle ear, it will be necessary to remove the whole of the petrous bone. For this purpose the incision behind the ear must be carried back along the anterior edge of the trapezius muscle halfway down the neck. Then the skin-flaps, including the external ear and the underlying tissues, must be dissected back for some distance on

each side of the incision. Two converging incisions are then to be sawn, the anterior passing through the root of the zygomatic arch, the posterior just back of the sigmoid sinus, so as to come together at the apex of the pyramid of the petrous bone, or, better still, to meet in the foramen magnum. An ordinary chisel and a hammer or mallet will be found very convenient for freeing the petrous bone after the incisions have been sawn.

In the examination of the petrous bone after it has been removed the first step is to chisel off the tegmen tympani so as to get a view of the middle ear. Next remove the lower wall of the external meatus, so as to expose the outer surface of the membrana tympani. Finally divide the petrous bone with a fine hair-saw by an incision starting in at the styloid process and coming out at the carotid canal, parallel to the crest of the pyramid of the petrous bone.

This incision divides the cavum tympani into halves. In the lateral half can be seen the membrana tympani with the hammer and the anterior half of the mastoid cells. In the median half are the labyrinthine wall of the cavum tympani with the stapes and the posterior half of the mastoid cells. It is best to remove the anvil before sawing through the bone. The Eustachian tube can be easily exposed by starting from its termination in the middle ear.

The Naso=pharynx.—Although a fair view of the nares and pharynx can be obtained by chiselling off the portion of the base of the skull lying over them, the method does not begin to offer the satisfactory view that can be obtained by the method of Harke,[1] a method which is not so difficult as it might at first sight seem, and which consists in halving the base of the skull by a longitudinal incision. To do this the original incision in the scalp must be extended on each side over the mastoid processes and along the anterior edge of the trapezius muscle to a point below the middle of the neck. Then the posterior flap and the underlying muscles must be freed from the occipital bone and the upper portion of the

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[1] *Berliner klin. Wochenschrift*, 1892, No. 30.

cervical vertebræ. In like manner, the anterior flap must be dissected from over the root of the nose and the upper edge of the orbits, and be drawn down over the face. Then flex the head strongly forward and saw through the occipital bone and the base of the skull, dividing the occipital and frontal bones, the sella turcica, the cribriform plate, and the basilar process into equal halves. Anteriorly, it is well to go a little to the left or right, so as not to injure the nasal septum.

The next step is to cut the pachymeninx and the apparatus ligamentosis between the anterior edge of the foramen occipitale magnum and the processus odontoideus, as well as the inner side of the atlanto-occipital joint from within. Then the two halves of the skull are to be drawn forcibly apart. The nasal bones, the hard palate, and the alveolar process of the upper jaw break, and the two halves of the base of the skull open like a book, revolving around an axis which passes through the joint of the lower jaw and the atlanto-occipital joint.

If the foramen occipitale magnum offer too much resistance, break through it with a chisel, and also if necessary through the anterior and posterior arches of the atlas.

It is now easily possible to inspect the sinus sphenoidales, the nasal septum, the frontal sinuses, and the nasal passages. The antrum of Highmore is easily opened with forceps and a pair of bone-shears.

After the operation the two halves of the base of the skull are brought together, and wired if necessary. When the skin-flaps have been replaced all evidence of the operation is covered up.

Examination of New=born and Very Young Children.—

1. The head is preferably opened by the method given on page 606.

2. According to Nauwerck, the spinal canal can be opened by dividing the vertebral arches with strong scissors.

3. The umbilical cord, if present, and the umbilical arteries demand close attention in children who have lived a few days or weeks, for the purpose of determining if infection has taken place at that point. Nauwerck advises a modification

of the primary long incision. A little above the umbilicus it should divide into two diverging incisions running to the pubes. In this way a triangular flap is left containing the umbilical arteries, while from the upper end is given off the umbilical vein. The vessels may be ligated or opened at any point that seems advisable.

4. Anomalies of circulation should be looked for in all "blue babies." The closure or non-closure of the *ductus Botalli* (arteriosus) is best determined *in situ* by dissecting off the thymus and opening up the pulmonary vein in the middle of its anterior surface. The cut may be extended downward, if desired, through the pulmonary valve and the wall of the right ventricle. The duct lies in the median line of the pulmonary artery, a little above its division into its two main branches. A small probe can be passed through it into the aorta. The condition of the *foramen ovale* between the auricles is easily examined.

For other anomalies of the circulation it will usually be found most satisfactory to remove the thoracic organs in mass, so as to be able to open up the heart and the vessels given off from it before any of the vessels have been severed from their connections.

5. In medico-legal cases especially it is important to determine whether or not a child has breathed. The main steps of the process are as follows:

(*a*) Position of the diaphragm before the chest is opened. When the lungs are fully distended it is at the fifth or sixth rib on the right and at the sixth rib on the left. When the lungs contain no air or are but partially distended the diaphragm reaches to the fourth rib.

(*b*) Ligate the trachea above the sternum before opening the thorax.

(*c*) After examining the heart, etc., divide the trachea above the ligature and remove the thoracic organs in one piece.

(*d*) Dissect off the thymus gland and the heart, and place the lungs in a large dish of clear cold water to see if they will float or not.

(e) Incise the lungs and notice if they crepitate; squeeze the lung-tissue gently, and see if bubbles of air mingle with the blood on the surface, or squeeze the lung beneath water and observe if bubbles of air rise to the surface. Decomposition may give rise to gas in the lungs.

(f) Divide the lungs into lobes, and then into small pieces, and determine if any of them will float.

TABLE OF THE WEIGHT AND LENGTH OF THE FETUS AT EACH MONTH OF GESTATION

(From v. Hecker, cited by Nauwerck)

Time in months	Weight	Length
2	4 gr.	2.5–3 cm.
3	5–20 "	7–9 "
4	120 "	10–17 "
5	284 "	18–27 "
6	434 "	28–34 "
7	1218 "	35–38 "
8	1549 "	39–41 "
9	1971 "	42–44 "
10	2334 "	45–47 "

6. The long bones should be incised so as to expose the epiphyseal line which should be examined for evidences of congenital syphilis. The ends of the femur and tibia at the knee are usually chosen. For making the incision a fine hair-saw is preferable to a knife, because the latter often causes the bone to break apart at the epiphyseal line.

The age of the fetus in months can be determined after the fifth month by dividing the length in cm. by 5.

WEIGHT OF ORGANS IN A NEW-BORN CHILD

Brain,	380 gr.	(Bischoff).
Thymus,	14 "	(Friedleben).
Heart,	20.6 "	(Thoma).
Lungs,	58 "	
Spleen,	11.1 "	
Kidneys together,	23.6 "	(Thoma).
Testicles,	.8 "	
Liver,	118 "	

Restitution of the Body.—After an autopsy is finished it is necessary to put the body into such a condition that no

evidence of the operation will be noticed except on careful inspection. All fluids should be removed from the cavities. Organs not required for further examination should be replaced. The brain is placed in the body-cavity because it is usually impossible to restore it to the skull. The best material for filling up the cavities is fine sawdust. It packs easily and smoothly, absorbs well, keeps the needle dry so that it does not slip, and does not interfere with sewing like oakum which gets into the stitches. In private autopsies any make-shift, such as bran, newspapers, or cloth, must be employed. If the pelvic organs have been removed, stuff the pelvis tightly to prevent leakage. The cranium may be left empty, although it is usually better to pack a little sawdust or other material into the base of the skull and the upper part of the spinal canal to prevent leaking. Sometimes it is advisable to fill the cranial cavity with sand or sawdust wrapped tightly in a cloth, of which the edges are brought together and twisted so as to crowd the material into a compact mass. If the thoracic cavity is well packed with sawdust, the sternum will stay perfectly in place without being sewed.

If part of the vertebral column has been removed, a stick or heavy iron rod should be run into the spinal canal above and below, so as to stiffen the body and hold it in position while it is filled about half full of plaster of Paris. After this has set there is little danger of the body losing its form.

In sewing up the body-cavity, begin at the neck. Use a piece of twine a little over one and a half times the length of the incision. Take one stitch and fasten the end with a simple knot or with a surgeon's knot. Turn the loose end in under the skin. Hold the attached end of the twine taut with the left hand about 8 to 10 cm. from the line of incision. The needle is then passed from within outward through the edge of the flap and in a diagonal line from below upward. The stitches should be from 1 to 2 cm. apart, and about the same distance from the edge of the flap. The object of keeping the end of the twine taut is to keep the sutures tight and the edges of the flaps up so that the needle can be thrust in easily.

Arrived at the lower end of the incision, take two button-hole stitches and draw them tight. Then take a long stitch off to one side and cut the twine close to the skin, so as to bury the end of it deeply and securely.

If in removing the calvarium the precaution is taken to crack at least a part of the inner table with the chisel and hammer, projecting pieces of bone are usually left which interlock and hold the calvarium snugly in position when it is replaced. It is further fastened by sutures on each side through the fascia of the temporal muscle. It is always more difficult to sew up the incision in the scalp than the one in the body, especially when the hair is long. Care should be taken to bury the ends of the suture securely.

The skull of a child is so thin that it is usually best to wire the calvarium in place or to fasten it by means of double tacks, otherwise it may slip out of place after the scalp has been sewed up.

Slee's ingenious method deserves mention. The usual saw-cuts in the skull over the ear are allowed to cross each other, so that slits about an inch long are formed in the temporal bone. An ordinary roller bandage is stretched across the skull and crowded edgewise into the slits. Then the calvarium is replaced and the ends of the bandage are tightly overlapped over the vertex and secured by pins.

List of Publications on Post=mortem Technique

1. Virchow, Sektionstechnik, 4 Aufl., 1893.

2. Orth, Pathologish-anatomische Diagnostik, VI Aufl., 1900.

3. C. Nauwerck, Sektionstechnick für Studierende und Aerzte, V Aufl., Jena, G. Fischer, 1912.

4. Chiari, Pathologisch-anatomische Sektionstechnik, Berlin, II Aufl., 1907.

5. G. Hauser, Die Zenkersche Sektionstechnik, Jena, G. Fischer, 1913.

POST=MORTEM EXAMINATION OF THE SKULL AND ITS CONTENTS IN THE NEW=BORN[1]

In view of the prominent place occupied by intracranial hemorrhage among the causes of death in the new-born (approxi-

[1] This section has been written by Dr. Donald Munro.

mately 35 per cent.), it is desirable that the method of post-mortem examination of the skull and its contents be such that an adequate examination may be made in gross, without at the same time causing post-mortem injury which might obscure or be confused with ante-mortem lesions.

An incision is made through the scalp down to the pericranium, or periosteum, extending from just behind one ear to just behind the other across the vertex. The scalp is then peeled forward to the supra-orbital ridges, and back to the superior curved line of the occiput. Hematomata in the scalp, sub-periosteal hematomata (the so-called cephal-hematomata) or other evidence of gross injury to the skull or its coverings are easily identified at this point.

Having completed the examination of the scalp and pericranium the skull should be opened as follows: At the right anterior border of the anterior fontanelle a sharp pointed scalpel is inserted through the fused periosteum and dura at their junction with the mesial edge of the right frontal bone. The point of the scalpel is directed laterally and is kept close to the inner surface of the right frontal bone in such a way that it penetrates between the bone and the dura, especial care being taken to prevent injury to the dura. A longitudinal slit in the suture line sufficiently large to admit the handle of the scalpel is made in this plane. The handle of the scalpel is then inserted through this slit between the bone and the dura and the two are gently and easily separated as far as is possible.

The frontal bone is then separated from the adjacent left frontal and right parietal bones by extending the original incision along the anterior portion of the sagittal suture to the level of the floor of the anterior fossa, and also along the fronto-parietal suture to the superior edge of the temporal bone. The right frontal bone having been thus isolated is turned outward and forward and broken along its base. A similar procedure is carried out with the right parietal bone, the underlying dura being separated as described above and the bone being isolated by incisions along the posterior portion of the sagittal suture, the parieto-occipital suture, and the parietal edge of the fronto-

parietal suture. The parietal bone is turned outward and broken at its base.

The removal of the frontal and parietal bones thus exposes intact the dura covering the right cerebral hemisphere. Epidural hemorrhages may be seen and should the question of an ante-mortem rupture of the middle menigeal artery be raised it is simple to expose this artery by removing the squamous portion of the right temporal bone according to the technique outlined above, following which, retraction of the intact dura mesially will expose the entire artery as far as its entrance to the skull through the foramen spinosum. The superior sagittal sinus should not have been opened.

Having inspected this portion of the dura while intact, it should then be incised longitudinally slightly to the right of the mid-line and in such a way that the superior sagittal sinus is not injured. The flap of dura, with the arachnoid usually attached to it, is then turned outward, vertical incisions being made at the anterior and posterior portions of the flap if necessary, and the right cerebral cortex exposed to view. Subarachnoid, subdural, or subpial collections of blood on this side should be noted as should also the condition of the cerebral veins in relation to the superior sagittal sinus and the parieto-occipital suture. Ante-mortem rupture of these veins is difficult if not impossible to identify positively, but a diagnosis of probable ante-mortem rupture may at times be made.

The gloved finger tip is now inserted between the mesial surface of the right cerebral hemisphere and the filmy layer of tissue which makes up the body of the falx care being taken to avoid tearing the latter. The corpus callosum is thus exposed. This should be carefully incised longitudinally in the mid-line and the cavity of the third ventricle be thus exposed to view. If this has been done with care the two internal cerebral veins and their point of junction to form the great vein of Galen will be plainly visible. With a rupture of the vein of Galen the two cerebral veins are collapsed and difficult to see, otherwise they are plainly visible and often are noticeably congested. The presence of intraventricular hemorrhage should

be ascertained by observation of the contents of the third ventricle.

Further incision through the floor of the third ventricle and the sub-thalmic region should be carried out at this point although the internal cerebral veins are thus destroyed. A lateral horizontal incision through the right cerebral peduncle and section of the right optic tract now frees the right cerebral hemisphere, which with a little urging will fall out into the operator's hand. The hemisphere may be examined at once or laid aside in some suitable solution to harden prior to later sectioning and examination.

Removal of the right cerebral hemisphere in this manner exposes the entire falx cerebri and its junction with the tentorium cerebelli as well as the right half of the latter.

The body of the falx should first be inspected for tears, hematomata or other lesions. This is best, and in fact can only be accomplished by lifting it free from the mesial wall of the left cerebral hemisphere on a scalpel handle or other suitable instrument. The lower free border containing the inferior sagittal sinus should next be inspected for ruptures. The junction of the falx with the tentorium should be observed with care especially if the two internal cerebral veins have been found collapsed, thus indicating a rupture of the great vein of Galen. Should this have occurred the posterior end of the ruptured vein may be present still attached by its junction with the straight sinus, or with the inferior sagittal sinus.

The right half of the tentorium should now be inspected. Ruptures of the free edge, often avascular and most commonly at or near its junction with the falx are frequent. Ruptures in any portion of the body may or may not involve the lateral sinus, the straight sinus or even the Torcular Herophili and may present themselves as frank holes, from which blood can be expressed—should they communicate with the cavity of a sinus—or may be indicated by an adherent ante-mortem clot, or may be present only as avascular tears.

The superior sagittal sinus should now be opened through its entire length and inspected for tears from its internal aspect.

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This will also expose the internal aspect of the Torcular Herophili.

The right lateral sinus may now be split along its course as also the superior and inferior petrosal, and the connection between lacerations in the tentorium thought to have extended into the cavity of one of these sinuses be thus verified.

The falx should now be freed along its upper border as far as may be convenient, turned to one side out of the way, the left cerebral peduncle incised horizontally, the left optic tract sectioned, and the left cerebral hemisphere allowed to drop out without further removal of bone.

The dura which previously covered this hemisphere may now be inspected from the inside, and the left middle meningeal artery observed. The left cerebral veins cannot be inspected except from the inner aspect of the superior sagittal sinus, and subarachnoid and subdural hemorrhages in this portion of the cerebrum will be evident chiefly as collections of blood on the cerebral cortex itself.

The anterior and middle fossæ with their contents may now be searched for fractures, hemorrhages or other lesions.

The left half of the tentorium cerebelli should be searched for evidence of injury either with or without hemorrhage and the left lateral sinus split throughout its length just as was done on the right side.

The tentorium should now be split in the mid-line toward the internal occipital protuberance and the two halves turned one to either side to expose the cerebellum and brain stem.

The cerebellum and brain stem are best removed in one piece in the following manner. The anterior border of the mid-brain is pushed gently backward off the body of the sphenoid bone and the cranial nerves are exposed, identified and sectioned pair by pair. In this way also the basilar and the two vertebral arteries are brought into view and their continuity verified. The posterior cerebral and posterior communicating arteries as well as the anterior half of the circle of Willis can not be saved.

Having freed the brain stem by section of all the cranial nerves, a thin bladed scalpel is inserted along the anterior aspect

of the medulla through the foramen magnum as far down the cervical cord as possible (usually not more than one or two segments) and the cord sectioned at this point. Occasionally it will be necessary, in addition, to bring the knife blade to the lateral aspects of the cord in order that the upper cervical roots may be cut before the cord will be found to be entirely free.

The cerebellum, brain stem and what little of the cervical cord it is possible to get may then be removed in one piece. If this is done with care, the roof of the fourth ventricle and the great basal cistern overlying it may be kept intact and later, after being suitably preserved and hardened, may be inspected for meningitis, obstruction of the foramina or other lesions.

The posterior fossa and foramen magnum are now exposed together with the occipital sinus which should be split, in its long axis, up to its junction with the Torcular Herophili.

The skull should now be filled with some appropriate material, the right frontal and parietal bones replaced, the scalp pulled back over them and the original incision sewed up.

According to Dr. Bronson Crothers the method of opening the head in such a way as best to disclose tears of the dura was originated by Beneke in 1910. It was elaborated by Meyer and Hauch in 1912 or thereabouts, and later rediscovered by Holland.

Beneke, R., *München. med. Wchnschr.*, 1910, lvii, 2125.

Meyer and Hauch, *Arch. Mens. d'obstet. et de gyn.*, 1912, i, 246.

Holland, Eardley, Causation of Fetal Death, Reports on Public Health No. 7 Ministry of Health, London, England.

ADDENDA

Method of Preparing the "Bacterial Vaccines" of Sir A. E. Wright.—These "vaccines" are suspensions of definite quantities of bacteria killed by heat, in a 0.9 per cent. solution of sodium chlorid. The method here described is a modification of Sir A. E. Wright's method as used in the Pathological Laboratory of the Massachusetts General Hospital.

As profuse a growth as possible of the bacterium is obtained in a number of "slant" culture-tubes, three or four tubes usually furnishing a sufficient mass of bacteria for the purpose. All of the bacterial growth in the tubes is collected in a thick suspension in sterile 0.9 per cent. saline solution in a sterile test-tube. This test-tube is then drawn out with the aid of a blast-lamp to a small diameter some centimeters above the level of the fluid, and is set aside to cool. When cool, the drawn-out portion is sealed off in the flame and the tube is thoroughly shaken during some minutes. The sealed extremity is then opened and a few drops of the suspension withdrawn into a small dish or onto a block of paraffin for the purpose of later determining the number of bacteria in suspension, after which the tube is again sealed in the flame. The suspension is now ready for sterilization. This is done by keeping the tube fully submerged in a water-bath at 60° C. for from one and a half to two hours.

The determination of the number of bacteria per cubic centimeter in the sample withdrawn from the sealed tube is made as follows:

The first step in the process is thoroughly to break up the clumps of bacteria so that each bacterium, as far as practicable is free and separate in the suspension. This may have been already accomplished by shaking the suspension in the test-tube, but if not, then the breaking up of the clumps may be

effected with the aid of a capillary pipette about 1 mm. in dia-
meter, prepared from a piece of glass tubing of about the
diameter used for the ordinary medicine dropper. To this
pipette is affixed a tightly fitting rubber bulb similar to that
used on a medicine dropper, but of the best quality of rubber.
The smaller end of the pipette must be squarely broken off.
The breaking up of the clumps is effected by repeatedly forcing
the bacterial suspension in and out of the pipette by manipulation
of the bulb, while the pipette is held perpendicularly against the
surface of the glass dish or paraffin block in such a way as to
bring as much as possible of the circumference of the smaller end
in contact with it, thus leaving minute clefts which are small
enough to cause the breaking up of the clumps as they are
forced through. In this process the pipette is most conve-
niently held in such a manner that the bulb may be manipu-
lated with the thumb and forefinger, while the remaining
fingers grasp the body of the pipette and steady it against the
surface of the glass dish or paraffin block. With some bacteria,
for example, the gonococcus, this procedure is not sufficient to
break up the clumps, and in this case the shaking of some cubic
centimeters of the suspension with fine sterilized sand in a small
tube is resorted to.

The next step is to determine the number of bacteria per
cubic centimeter in the suspension. This may be done in
either of two ways.

One way is to mix thoroughly equal quantities of freshly
drawn normal blood, of a fluid which prevents the coagulation
of the blood, and of the suspension; then in stained smear
preparations of the mixture determine the ratio between the
number of red blood-corpuscles and the number of bacteria.
Assuming five million red blood-corpuscles to a cubic millimeter,
the number of bacteria per cubic centimeter is readily
determined.

This procedure is carried out with the aid of a capillary
pipette provided with a rubber bulb like the pipette described
above. The mark is made on the pipette 2 or 3 centimeters
from its smaller extremity, and into the pipette, while grasped

in the hand, as before described, there is drawn up to this mark successively the fresh blood, the anti-coagulating fluid, and, finally, the bacterial emulsion, a small amount of air being allowed to enter the tube after each measure of fluid, and the end of the pipette wiped after each taking. The contents of the pipette are immediately expressed onto a glass dish or paraffin block, and the elements in the various fluids thoroughly mixed by drawing the mixture in and out of the pipette repeatedly. Smears are then prepared and stained with Wright's blood-stain, as in the case of blood-smears. The counting is done under an oil-immersion objective with an eye-piece, upon the inferior lens of which a square has been marked out about 9 mm. on a side, with a wax pencil. The number of red blood-cells and bacteria seen within this ruled square are counted in various portions of the preparation until 1000 red cells have been counted. The anti-coagulating fluid employed consists of 1.5 per cent. sodium citrate in 0.9 per cent. sodium chlorid solution.

Another way of determining the number of bacteria per cubic centimeter in the emulsion is to count the bacteria without staining in a chamber similar to the Thoma-Zeiss blood-counting chamber. This method has been devised by one of us, and is regarded as much easier of execution than the one above described. The chamber used is manufactured by Zeiss for counting blood-plates by the Helber method. It should be supplied with an especially thin cover-glass (No. 146, Zeiss' Catalogue) to permit the use of the high-power dry objective with which the counting is made. The chamber is ruled like the "Thoma-Zeiss blood-counting chamber," and the rulings have the same value, except that the chamber is 0.02 mm. deep instead of 0.1 mm. For counting, the suspension of bacteria is diluted and mixed with distilled water 1 : 200 with the aid of the red blood-corpuscle pipette of the "Thoma-Zeiss" apparatus. By a simple calculation it will be apparent that the product of the multiplication of the average number of bacteria per small square by 4000 million will be the number of bacteria per cubic centimeter.

When the heating of the suspension is finished, the small end of the sealed tube is broken and a "planting" made from the emulsion upon the surface of a blood-serum "slant" to test the sterility of the emulsion. Immediately after this a sufficient quantity of the emulsion is mixed with sterile 0.9 per cent. saline solution to give a dilute suspension of the volume of 50 c.c. containing the required number of bacteria per cubic centimeter. This is done as follows:

A small flask containing 50 c.c. of 0.9 per cent. of saline solution, closed with a rubber nipple, and the whole sterilized, is previously prepared. The quantity of the suspension necessary to give the desired number of millions of bacteria per cubic centimeter in a volume of 50 c.c. of saline solution having been determined by calculation, this quantity is withdrawn from the flask by means of the sterilized hypodermic syringe, the needle of the syringe being plunged through the rubber nipple while the flask is inverted; then the calculated quantity of the suspension is drawn up into the syringe and injected into the saline solution by passing the needle through the rubber nipple as before. Following this, 0.15 c.c. of lysol is similarly injected into the flask through the rubber nipple, and, after shaking, this diluted suspension constitutes the vaccine. Before injecting it, it should have been proved sterile.

The vaccines prepared from the staphylococci are made up so as to contain 600 million staphylococci in each cubic centimeter, while those of other bacteria are made up to contain only 100 million.

The dose varies according to the circumstances of the case. The full dose of the staphylococcus vaccine is 600 million, while a full dose of other vaccines is 100 million. The injection is made subcutaneously, usually in the abdominal wall in men and between the shoulder-blades in women, these being readily accessible and less sensitive areas. In charging the syringe for the injection, the needle is passed through the rubber nipple with the flask inverted. Before doing this, the surface of the rubber nipple should be sterilized, either with lysol, or by plung-

ing the nipple and neck of the flask into hot water for a few seconds.

Methyl=violet Shellac.—

Best white shellac,	10 gm.;
Alcohol, 95 per cent.,	20 to 25 c.c.;
Methyl-violet,	0.1 gm.

This solution will be found very convenient for marking important fields in mounted sections. It may be used with the circular markers made for this purpose, but a pen is just as convenient and less liable to cause injury to the preparation by pressure. The desired field is readily outlined under the low power of the microscope by a series of dots or a continuous line. The solution after drying is insoluble in xylol or water.

METHOD FOR BLACKENING TABLE TOPS
Solution I.

125 grams copper sulphate ⎫
125 grams potassium chlorate ⎬ Boil until dissolved.
1000 c.c. water ⎭

Solution II.

150 grams anilin hydrochlorate,
1000 c.c. water.

Use white wood for tops, since it is cheaper and takes the stain better than pine. Oak gives good results.

Spread papers to cover floor completely under and around the tables.

1. Apply with brush two coats of Solution I *while hot;* the second as soon as the first is dry.

2. When second is dry, apply in the same way two coats of Solution II and let them dry thoroughly.

3. Put on with a *cloth* a thin coat of raw linseed oil and polish thoroughly by rubbing.

4. When dry wash thoroughly with *hot soapsuds*.

5. Rub again with raw oil.

6. Rub with cotton waste over and over again until the black does not come off. This requires hard work.

To keep tops in perfect condition, rub off once a week with white (liquid) petrolatum.

N. B.—If table tops are blotched with paraffin, wax, or resin, remove these before beginning to paint. Stains do not harm. Paint top surface and edges only.

TO CLEAN SLIDES

For ordinary purposes new slides and cover-slips are cleaned by dipping them in alcohol and wiping with a soft crash towel or old linen handkerchief.

A better but more complicated method is to soak them for one or more hours in the following cleansing solution:

Bichromate of potassium,	8 grams;
Sulphuric acid,	12 c.c.;
Water,	100 "

in order to remove any alkaline silicates which may have formed on the surfaces, and then to wash thoroughly in water, dip in alcohol and wipe as before.

Cover-slips, after they are clean, should be preserved dry in covered dishes. The common method of keeping them under alcohol cannot be recommended.

To clean old slide preparations, heat them until the balsam softens so that cover-slips and slides can be drawn apart. The slides and cover-slips are then treated separately. The cover-slips are soaked first in xylol and then in alcohol for several days to remove the balsam, left over night in a strong solution of one of the powdered soaps (*do not boil them*), washed thoroughly in water, dipped in dilute acid or in acid alcohol, washed again in water and wiped from alcohol.

The slides are put into jars of waste alcohol, or of xylol followed by alcohol, for several days, and then treated in the same way as the cover-slips; namely, soap solution, water, acid solution, water, alcohol followed by wiping.

Hand Lotion

Gum tragacanth,	9 to 10 gm.;
Boric acid,	20 "
Glycerin (double distilled),	50 c.c.;
Water,	ad 1000 "
Oil of rose geranium,	1 "

Mix the first four ingredients in a bottle, and place in a warm place. Shake occasionally. After one to four days (whenever the gum is thoroughly softened) filter or squeeze through fine cheese-cloth or a towel, on the dry surface of which the oil of geranium has been poured. If preferred the oil can be dissolved in a little alcohol and added directly to the mixture. The amount of glycerin can be increased if desired.

PHOTOGRAPHS OF GROSS PATHOLOGICAL SPECIMENS

Preserved organs and tissues are as a rule to be preferred, but good results can be obtained with fresh material, even if taken under water, if all free blood is first carefully washed off.

For dry specimens the same procedures hold as for photographs in general; hence no special directions are required. Wet specimens, however, require different treatment owing to the presence of annoying high lights.

Several simple but important rules must be followed in order to obtain good photographs of pathological specimens in either the dry or the wet condition. They are as follows.

1. Focus on the surface of the specimen. If no sharp details are present place a printed card on the highest point and focus on that. Then close the diaphragm to a small opening (F.32–F.64). This procedure greatly lengthens the time of exposure required but gives sharp detail and great depth of focus.

2. Avoid all shadow around the specimen by removing the background to a distance so that the shadow disappears.

3. Use a white, gray or dark background according to the color of the tissue which should be made to stand out in sharp contrast with it.

4. Avoid all high lights by wiping dry all glistening points of a wet specimen or, much better, by placing the object to be photographed, if not too large, under water in a glass tank with glass bottom and using a vertical camera.

5. Illuminate the specimen chiefly from one side, and somewhat obliquely, so that the projecting points will cast a little shadow.

6. If the background is white it may require special illumination in order to light it up strongly and evenly.

Artificial illumination (a Mazda light, 500 to 1000 Watts with reflector back of it) is in general to be preferred to daylight because it is constant in quantity and is always available.

For taking pictures of specimens under water the tank with glass bottom and sides devised by T. Bitterman[1] can be highly recommended. Its dimensions are, length 25 inches, width 16 inches, height $6\frac{1}{2}$ inches. It is elevated on legs 10 inches high, in order that the shadow cast by the specimen may be carried out of the field, and has a small hole drilled through one corner of the bottom to facilitate removal of the water.

Bubbles of air which are deposited on the glass bottom from the water must be rubbed away, otherwise they will show in the finished print.

We have obtained excellent results, using the slow No. 23 Seed double coated plates. Bitterman recommends the "commercial orthonon films" supplied by the Eastman Kodak Co. When a filter is necessary he uses the panchromatic plate and the Wratten and Wainwright K3 filter.

THE ESSENTIALS OF PHOTOMICROGRAPHY

The first and greatest secret of good photomicrographs is perfect sections perfectly stained. If one has them the rest of the process is comparatively simple.

The second important point in photomicrography is choice of field. This is a time consuming process but it is absolutely

[1] The Photography of Gross Pathological Material, Major T. Bitterman, *Bulletin* No. viii of the International Association of Medical Museums and *Journal of Technical Methods*, 1922.

necessary if the picture is to tell its story.   No artefacts of any sort, such as scratches or foreign bodies, should be tolerated. The selected fields should be circled either by means of a diamond marker or with a pen dipped in methyl violet shellac. When it comes to photographing the desired fields they should be rotated by means of the movable stage until they present a well balanced appearance with the main lines running horizontally or vertically and with the most important feature in the center of the field.

In photographing a series of slides it is advisable to do at one time all the fields requiring the same magnification.   By this method the timing of the exposures is simplified and rendered a certainty and the changes of lenses, eye pieces and condensers reduced to a minimum.   It will be found that as a rule it is much easier to take good high power pictures than those of low magnification.

The following staining methods all give good results photographically.   An intense, sharp stain is desired but it should not be too deep except when very low magnification is wanted.

(*a*) Eosin and methylene blue after Zenker fixation; the best general stain and the one most commonly useful.   Under this heading should be included the Giemsa and Romanowsky stains.

(*b*) Alum hematoxylin and eosin; used chiefly for celloidin sections.   The resulting prints and lantern slides have a soft attractive quality.   The hematoxylin alone is useful for bringing out the nuclei in Gram-Weigert and tubercle bacillus stains.

(*c*) Phosphotungstic acid hematoxylin; excellent for tumors, especially when mitotic figures and neuroglia, fibroglia or myoglia fibrils are present.

(*d*) Weigert's and Verhœff's elastic tissue stains; the latter is especially designed for tissues fixed in Zenker's fluid.

(*e*) Gram-Weigert and tubercle bacillus staining methods.

Photomicrographic Apparatus.—We are acquainted only with the Zeiss outfit which includes apochromatic lenses and compensating oculars.   It can be highly recommended.   Several other makes are on the market.   Whatever one is available must

be thoroughly studied and understood if it is to be used to the best advantage. The points to which especial attention must be paid are the leveling and alignment of the apparatus; the adjustment and centering of the arc light; the system of condensers used and their position relative to each other and to the microscope in order to produce the best lighting effect; the Abbe and simple substage condenser; the microscope and its lenses. If these are not all properly adjusted the pictures often show a peculiar blurring effect (halation?) which is displeasing.

It has been our experience and that of three or four others using similar outfits that better low power pictures can be obtained with a selected Leitz No. 3 achromatic objective and No. 1 eye piece than with the Zeiss apochromatic No. 16 objective and No. 4 compensating ocular. All the other Zeiss apochromatic objectives give excellent results as do also their planar lenses for very low powers.

Color Screen.—We have never used but one (to the despair of visiting salesmen with elaborate sets of color screens to sell) and it works perfectly well with all the staining methods mentioned above. It is the following:

Copper sulphate,	175 grams;
Potassium bichromate,	17 "
Sulphuric acid,	2 c.c.;
Water up to,	500 "

It is used in a flat glass cell which is vaselined within along its upper edge and then sealed with surgical plaster to prevent evaporation.

Plates.—Cramer Isochromatic Slow. They were selected after much experimentation and have always given good results.

Developer for Plates:

SOL. A

Water,	1000.0 c.c.;
Pyrogallic acid,	20.0 grams;
Potassium metabisulphite,	2.5 "

SOL. B

Water,	1000.0 c.c;.
Sodium sulphite (anhydrous),	100.0 grams;
Sodium carbonate (anhydrous),	40.0 "

Use equal parts for developing plates. Add ten to twelve drops of a 10 per cent. aqueous solution of potassium bromide to 120 c.c. of mixture.

This is a strongly alkaline pyrogallic mixture. A properly exposed plate is fully developed by it in three minutes. As soon as the developer is poured over the plate brush the surface gently with a wad of cotton to remove all air bubbles. Go over the surface in the same way after the plate is washed and just before it is put to dry in order to remove any particles of dirt or iron rust in the running water which may have been deposited on it. It is desirable always to err on the side of underexposure as this fault is more quickly appreciated and corrected than overexposure.

Fixative for plates and prints:

A

Water,	3840 c.c.;
Sodium hyposulphite,	960 grams.

B

Water,	960 c.c.;
Sodium sulphite (anhydrous),	90 grams;
Sulphuric acid,	15 c.c.;
Chrome alum (powdered),	60 grams.

Pour B into A while stirring well. Fix about twenty minutes.

This is the acid chrome alum fixing solution in general use by professional photographers. It keeps indefinitely in stock solution.

Printing Paper.—Normal Glossy Cyko. We have found this paper very satisfactory for our negatives but other glossy papers on the market may give as good or possibly even better results. Only trial will tell.

Developer for prints:

Metol,	1.0 gram;
Hydrochinon,	3.5 grams;
Sodium sulphite (anhydrous),	24.0 "
Sodium carbonate (anhydrous),	24.0 "
Potassium bromide,	1.3 "
Water,	1000.0 c.c.

As is well known the metol sometimes causes eczema on the fingers but with a little care or by the use of rubber cots this can usually be avoided.

Squeegeeing.—After the prints have been fixed and washed they are squeegeed on to the surface of ferrotype plates.

To prevent sticking, a few drops of a *saturated solution of white beeswax in turpentine or in xylol* are poured on the plate and then the surface is rubbed with a soft towel until it is highly polished.

As prints prepared in this way often show a moth eaten appearance on the surface where particles of air have prevented intimate contact with the plate we have found it advisable to dip the prints *in 25 per cent. alcohol* just before squeegeeing them.

Old prints can be treated in the same way if they are first soaked in water. As soon as the prints come off the plates they should be put under pressure (between the leaves of a book will do) to prevent curling. If they have curled at all badly hold them over a steaming dish of hot water for a moment to soften the surface and then run the prints over the edges of a desk or table, with the print surface uppermost to reverse the curl and then put under pressure again until perfectly dry.

Lantern Slides.—We have used various makes and have found most of them satisfactory.

Developer for lantern slides:

Water,	1000.0 c.c.;
Hydrochinon,	6.25 grams;
Sodium sulphite,	11.25 "
Citric acid,	.7 "
Potassium bromide,	.7 "
Sodium carbonate (anhydrous),	50.0 "

Dissolve in order listed. Develops slowly at first, rapidly later.

Do not overexpose; develop up full; make exposures in an enlarging and reducing camera, not by direct contact.

A working knowledge of photography is a desirable asset before undertaking photomicrography, but with persistence and experience anyone can in time expect to get fairly good results.

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